PARASITOLOGY

LONDON
CAMBRIDGE UNIVERSITY PRESS
FETTER LANE, E.C. 4

CHICAGO: THE UNIVERSITY OF CHICAGO PRESS
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PARASITOLOGY

(Founded by G. H. F. Nuttall, F.R.S.)

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VOLUME XXIX 1937

CAMBRIDGE
AT THE UNIVERSITY PRESS
1937

PRINTED IN GREAT BRITAIN

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Japan

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VOLUME XXIX JANUARY 1937 No. 1

RECENT PROGRESS IN THE CURE OF BILHARZIA DISEASE IN SOUTH AFRICA

BY F. GORDON CAWSTON, M.D.

A CONSIDERATION of the therapeutic doses generally used by South African doctors who avoid toxic effects during the treatment of schistosomiasis indicates that a permanent cure of the disease may be effected with either the potassium salt or the sodium salt of antimony tartrate within a month. A dose of 2 grains need never be exceeded with antimonium potassium tartrate in fresh solution as a maximum repeated dose. A total of 20 grains of this salt is a sufficient total dose; but the solutions of the drug should not be kept. The sodium salt of antimony tartrate was found less stable than the potassium of this compound.

The larger doses of the sodium salt which have been given in the past indicate that sufficient consideration has not been paid to the antimony content of the compounds. According to the *British Pharmacopoea* for 1932 there is a difference in the antimony content of only 0·42 m./mg. in the minimum dose and 1·62 m./mg. in the maximum repeated dose.

Compound	Formula	Molecular wt.	Antimony metal	*British Pharmacopoea* 1932 doses	
Potassium antimonium tartrate	$C_4H_4O_7Sb\,K\,\frac{1}{2}H_2O$	333·9	36·46% at 100% purity	10·93 m./mg. stibium	43·75 m./mg. stibium
Sodium antimonium tartrate	$C_4H_4O_7Sb\,Na$	308·8	37·85% at 96% purity	11·35 m./mg. stibium	45·42 m./mg. stibium

The potassium salt therefore contains a higher proportion of antimony metal, and the efficacy of the drug employed for treating this infection would seem to depend very largely upon its antimony content.

The total dose required varies to some extent with each patient and the amount of infection, but cases of long infection often respond to treatment with smaller total doses than fresh infections, and patients harbouring numerous parasites tolerate larger doses than others. *Schistosomum mansoni* and *S. bovis* are both stouter parasites than the more common *S. haematobium* and require a relatively higher total dose. This explains why the total dose commonly employed in Egypt, where infection with *S. mansoni* is commoner than in South Africa, is about 5 grains higher than is needed in South Africa. I have noted a slight increase in the proportion of lateral-spined ova towards the end of treating cases of mixed infection, and have observed similar signs of degeneration in the three types of ova whilst patients have been under treatment with emetine and the antimony compounds.

The possibility of producing antimony-fast parasites must be considered in patients who are given incomplete courses of treatment. Special efforts to administer a complete course must be made in these cases and, as cases of reinfection or recent cases also require a full course, the eosinophile count may be a useful guide to the dose required, for recent cases reveal a strong eosinophilia.

Experience would show that the vast majority of persons receiving fouadin are incompletely cured of bilharzia infection in South Africa. Due consideration has not been paid to the fact that this easily applied compound contains only 13·5 per cent of antimony metal instead of 36·46 per cent as contained in tartar emetic. To reach even half a gram of antimony metal about 60 c.c. of fouadin are required. This conclusion, reached by investigating the proportion of metal in antimony derivatives, is borne out by clinical experience.

The secretary of a hospital board in the Transvaal states: "It appears that the drug", tartar emetic, "is the only sure cure for bilharzia in the long run. During the last ten years about thirty-five patients have been treated with fouadin in this hospital and, invariably, the cases have returned as uncured after a month or two. About two years ago seventy-five school children were given tartar emetic injections as laid down by the Department of Public Health. These cases were all cured permanently."

A doctor at Middelburg, finding the recommended doses of fouadin ineffective, has used a total dose of 70 c.c. and, judging from the absence of escaping ova, he has found this to suffice.

At Barberton relapses occurred after fouadin injections until a second or a third course was given. I have found little conclusive evidence of the destruction of both male and female parasites in reported cures from fouadin, but Dr George Buchanan of the South African Institute for Medical Research tells me of one case where the blood count returned to normal and the symptoms disappeared after a third course of fouadin injections.

Those practitioners who employ intravenous injections of antimony experience uniformly good results. At Zeerust intravenous injections of antimonium sodium tartrate have been successfully applied since 1917, so that the town itself has been cleaned up of the infection, though bilharzia disease remains a factor of greater importance in the district than either syphilis or tuberculosis. As in other parts of the Union, uretral calculus and chronic appendicitis are here common complications. Many patients who do not complain of haematuria are unlikely to have their condition diagnosed until the characteristic ova are recovered from pathological material obtained by operation.

The late Prof. Sandwith pointed out that the eosinophile count sometimes rose to 52 per cent in a bilharzia patient but was found as low as 2 per cent. Dr J. B. Christopherson also points out that the eosinophile count is not an unfailing guide but that a rise during treatment is common. This temporary rise may be regarded as evidence of successful treatment.

Five Zulu boys harbouring *S. haematobium* revealed an eosinophile count of 7, 9, 11, 13 and 15 per cent. An Indian at Mariannhill had an eosinophilia of 18 per cent before treatment, but in another case the eosinophile count was only 2 per cent.

A high eosinophilia suggests a skin infection or recent infection. Mixed infection also causes a high eosinophilia, even in the chronic stage. An eosinophilia of 39·5 per cent. in my own case before treatment and persistent enlargement of the liver with abdominal symptoms suggested infection with *S. mansoni*, though only terminal-spined ova were ever recovered. Under the Streatfeild Research Trust I administered a total of 13 grains of freshly dissolved tartar emetic in 23 days to a boy of 13 harbouring both *S. haematobium* and *S. mansoni*. Towards the end of treatment his eosinophile count was 20 per cent, 19 days after treatment it had fallen to 7 per cent. Ova were absent from the urine on several subsequent occasions and I obtained a negative complement-fixation test, but a larger total dose would have been advisable.

Among Europeans in Natal, chronic patients often show an eosinophile count of 6–12 per cent, but those who harbour the infection for many years reveal a smaller proportion, and one patient who had not received any treatment during 10 years' infection revealed an eosinophile count of 4 per cent.

Emetine is still used to control the haemorrhage where a large number of natives are infected, and efforts to cure cannot readily be arranged. As an illustration of the incidence of the disease among natives, I recently found twenty-seven out of fifty-eight juvenile native delinquents at Durban infected with *S. haematobium*.

Any case will improve from injections of preparations containing antimony. Antimony thioglycollamide cannot be advised for curing the disease because of its low antimony content and comparative insolubility. About 1·7 g. would be needed before ½ g. of the metal is reached.

Should there be any reason to doubt that a cure has been effected by other remedies, there is no objection to the course being completed with intravenous injections of tartar emetic. However, the custom of discontinuing treatment of bilharzia disease until there is reason to suppose that all the parasites have been destroyed is unsound and is liable to account for appendicitis and other pathological conditions from incompletely destroyed worms which no longer give rise to haematuria.

The association of schistosomiasis with filaria infection must not be overlooked, and the beneficial effect of antimony in some cases of ascites suggests that filariae may be present in the night blood.

(*MS. received for publication* 11. I. 1936.—Ed.)

A PARASITE OF *SCIUROPTERUS VOLANS* L.

By V. REDIKORZEV

Academy of Sciences, Leningrad

(With 1 Figure in the Text)

Some time ago I received for identification a microscopic preparation of parasites taken, on 11 March 1929, from a flying squirrel (*Sciuropterus volans* L.) in the neighbourhood of Agraz, Tartar Republic. The preparation mounted in Canada balsam contained one male and two females. Later I examined a rather large collection of flying squirrels in the Division of Mammals of the Zoological Institute of the Academy of Sciences of U.S.S.R. In one jar containing a flying squirrel from Verchoijansk, Yakutia, 67° 33′ N, 133° 51′ E, I found, among the debris on the bottom, a single specimen of the same parasite.

The parasites were sent to me under the name of "mites", and at first sight they closely resembled a mite allied to the genus *Myobia* which usually parasitize small rodents. After a short examination I found that the preparation contained insects, not mites. Fig. 1 shows the exact form of the insect, but the examination and drawing of some of its characters proved to be difficult.

At first sight the insect seems to be headless, as its head, pro- and mesothorax are bent back, but whether onto the dorsal or the ventral side is difficult to decide in preparations. Thus the animal appears as if folded in two. Since such a position of the fore part of the body is to be seen in all four specimens obtained from different localities, it is to be regarded as normal for the insect.

The antennae (*a*) are strongly developed, their dimensions being not inferior to those of the legs; they are five jointed and geniculate, with the apical joint much widened and provided with spine-shaped setae.

The three pairs of legs (1, 2 and 3) are decreasingly less developed from the first to third. The first and second pairs are very similar to one another in structure and differ only in their dimensions. The femora and tibiae of all the legs are very short and have only a few short setae; the tarsi of the first two pairs are much widened, and have rather long claws and a row of short spine-like setae; the tarsi of the third pair are terminated with a large hook-like claw, with an opposite appendage on the apical joint. One of the specimens of the preparation has the right leg clasping a piece of hair of the flying squirrel. Thus, it is evident that the third pair of legs of this insect are the main organs of attachment to the hairs of the host. In general, the legs are short and do not project from under the body of the insect.

The boundaries of the abdominal segments are almost indiscernible and may be determined only by means of their chaetotaxy. The setae are very scanty and are concentrated mainly on the dorsal surface and on the pleural plates of the body. The setae are similar, being slightly enlarged in the middle

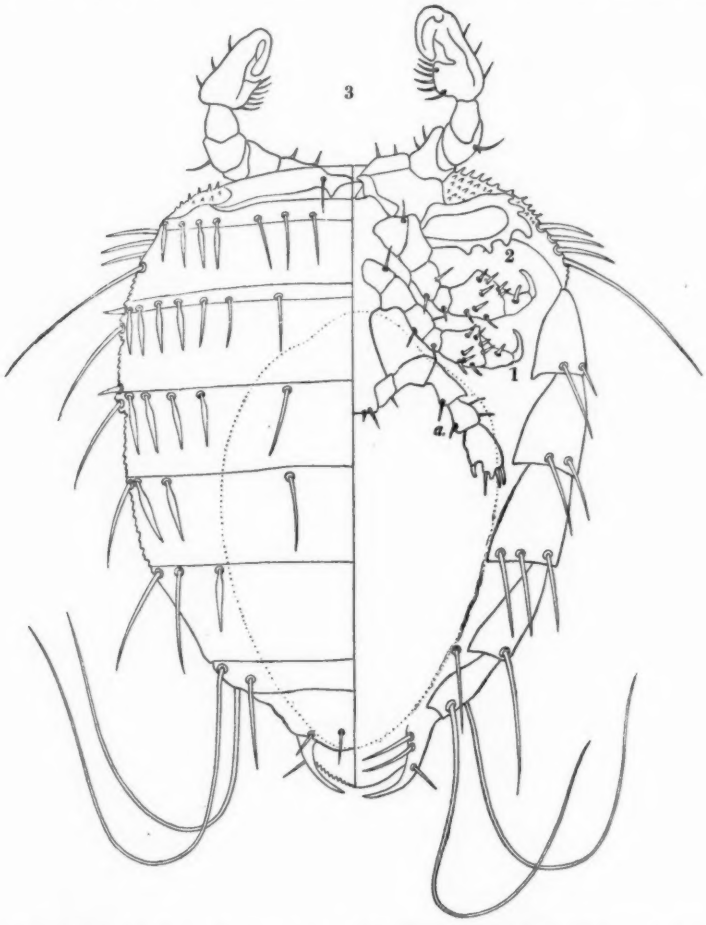

Fig. 1. *Enderleinellus replicatus*, sp.n., female; dorsal (on the left) and ventral view (on the right). *a*, antennae; 1, 2, 3, first, second and third pair of legs.

with sharp or spear-like apices. They are located in transverse rows on the tergites and number 7, 7, 5, 3, 3, 2 and 2 on tergites 1–7 respectively. The front margin of the abdomen bears three short ventral setae on both sides; the notum has but one much longer seta. The pleurites on their inner margins are not denticulated; the inner ventral tooth may be seen only on the first

pleurite. The posterior margins of pleurites 1–5 bear 2, 2, 3, 1 and 1 spine-like setae respectively, the last of which is very long, like its nearest dorsal seta.

Each side of the apex of the female abdomen bears a sharp hook-like appendage, with two setae on its base.

The integument of the body has a scale-like structure, the distal part of the scales being slightly raised; this feature may be seen easily from the side, when the scales are viewed in profile. The anterior part of the abdomen is covered with small spinules.

On the ventral side of the metanotum, laterally, is placed a strongly chitinized ring-like structure with six different obtuse spines on its margin.

The abdomen of the female contains an exceedingly large egg, shown in the figure by the dotted line.

Measurements: male 0·368 × 0·288 mm., female 0·352 × 0·320 mm.

The structure of the mouth-parts, the legs, especially their claws, the pleurites, the apex of abdomen, the shape of setae and their arrangement, the size and the form of the egg, are all characters which connect this very interesting insect with the order Anaplura.

I name this insect *Enderleinellus replicatus*, sp.n., belonging to the family Haematopinidae.

(*MS. received for publication* 12. II. 1936.—Ed.)

ON A NEW COCCIDIUM, *EIMERIA RAIARUM* SP.N., WITH SPECIAL REFERENCE TO THE BIOLOGY OF THE OOCYSTS

By LOUIS VAN DEN BERGHE, M.D., Sc.D.

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(With Plate I and 1 Figure in the Text)

During my stay at the Zoological Station of Wimereux, Pas de Calais, France, in August 1934, I made a special study of Protozoa parasitic in marine fishes. The examination of ten specimens of young *Raia batis* revealed a coccidial infection in two individuals, measuring about 25 cm. in length. In one of these the mucus in the terminal portion of the intestine contained a few unsegmented spherical oocysts, with diameters from 24 to 28 μ. Sections of different portions of the intestine and of the liver failed to exhibit any stages of schizogony or gametogony, evidently owing to the fact that there was a single infection at the end-phase. However, the second skate showed very numerous unsegmented oocysts in the posterior portion of the alimentary canal (Pl. I, fig. 1). This specimen provided material for the morphological study and biological observations which follow.

I. Description of the parasite

(1) *Exogenous development.* The oocysts with an unsegmented zygote (Pl. I, fig. 2) are more or less spherical, measuring from $20{\cdot}0 \times 17{\cdot}5\,\mu$ to $26{\cdot}2 \times 23{\cdot}0\,\mu$, the average being $22{\cdot}4 \times 20{\cdot}0\,\mu$. The wall is very thin and colourless, apparently without a micropyle. After remaining in sea water not more than 12 hours, the zygote gives rise to four ovoid sporoblasts with granular cytoplasm, leaving a large oocystic residue with a diameter from 10·5 to 12·4 μ (Pl. I, fig. 3). After one or more days the sporoblasts become invested with a sporocyst of an ovoid, subspherical shape, measuring about $7{\cdot}0 \times 6{\cdot}2\,\mu$. Each of the four sporocysts gives rise to two club-shaped sporozoites, with a very small, almost indistinguishable sporocystic residue.

(2) *Endogenous development.* The endogenous cycle of development of this parasite takes place in the intestine of the skate. The liver and the entire intestine were fixed and sections made from various portions. Gametogony was seen only in the epithelial cells of the last third of the intestine. The infection was at its end, so that the early schizogony stages were absent. The young female gametocytes are ovoid bodies, measuring about $16{\cdot}2 \times 10{\cdot}8\,\mu$, with the darkly stained globular peripheral inclusions characteristic of

developing coccidian macrogametocytes. When these reach maturity they are either elongated, measuring about $17{\cdot}8 \times 12{\cdot}6\mu$, or irregularly rounded, $18{\cdot}0 \times 16{\cdot}2\mu$, with a nucleus of $1{\cdot}8\mu$. The stages in Pl. I (figs. 4 and 7) are either late macrogametes or early zygotes, possibly with a thin oocyst wall. In these stages the globular inclusions disappear, being used up in the formation of the oocyst wall. The young microgametocytes are spherical or ovoid, the former type having a diameter of about $12{\cdot}6\mu$ and the latter measuring about $16{\cdot}7 \times 10{\cdot}0\mu$. They contain numerous compact nuclei filling up the entire body of the parasite (Pl. I, fig. 5). The adult microgametocytes are sometimes very large, measuring from $22{\cdot}0 \times 14{\cdot}6\mu$ to $26{\cdot}2 \times 126\mu$. Pl. I, fig. 6, shows fully developed microgametes and the gametocytic residue in the vacuole of a host cell.

As far as I am aware no coccidia of the genus *Eimeria* have hitherto been recorded from skates, while only the following four species were described from other elasmobranch fishes:

(1) *Eimeria lucida* Labbé, 1893: in *Acanthias*, *Scyllium catulus* and *Mustellus canis* (oocysts $10 \times 11\mu$).

(2) *Eimeria gigantea* Labbé, 1896: in *Lamna cornubica* (oocysts $70 \times 40\mu$).

(3) *Eimeria scylii* Drago, 1902: in *Scyllium* sp. [Unfortunately, I was unable to consult the original description of this species (*Ric. Lab. Anat. norm. Univ. Roma*, **9** (1902), 89), nor is it referred to in Wenyon's *Protozoology*, or by Halawani, in conjunction with his *Eimeria southwelli*.]

(4) *Eimeria southwelli* Halawani, 1930: in *Aetobates narinari* (very polymorphic elongated oocysts varying in length from 12 to 38μ).

With the exception of *Eimeria scyllii* none of the species from the elasmobranchs corresponds to the form described in the present paper.

Although the host specificity of all coccidia is very narrow, precautions were taken to eliminate the possibility of accidental infection of the skates by *Eimeria* from some other hosts, since the two infected skates were kept for about 2 months in company with various other fishes in a large aquarium. All these fishes (3 *Conger conger*, 1 *Cottus bubalis*, 5 *Onos mustela*, 6 *Pleuronectes platessa*, 6 *Pholis gunnellus*, 4 *Callionymus lyra*) were, therefore, sacrificed and examined, but none of them proved to harbour any coccidia. In view of these facts I propose to designate the new parasite of *Raia batis* as *Eimeria raiarum* sp.n.

Diagnosis. *Systematic position*. *Eimeria raiarum* sp.n. (Coccidiida Eimeriidae).

Description. Oocysts $20{\cdot}0 \times 17{\cdot}5\mu$ to $26{\cdot}2 \times 23{\cdot}0\mu$; sporocysts $7 \times 6{\cdot}2\mu$. Unsegmented oocysts discharged from host.

Host. *Raia batis* (Pisces Selachii).

Habitat. Posterior portion of the intestine within the epithelial cells.

Locality. Wimereux, Pas de Calais, France.

II. BIOLOGY OF THE OOCYSTS

It is generally accepted that "in cold-blooded animals the oocysts complete their development before leaving the host, whereas in the case of warm-blooded animals development takes place outside the body" (Wenyon, 1926, p. 830), though Wenyon (*loc. cit.*, p. 809 *et seq.*) himself described the complete development *in vivo* in *Isospora bigemina* of cats and dogs.

In the case of *Eimeria raiarum* the oocysts were all unsegmented within the alimentary canal, a fact which could be established by examination of practically all the oocysts present, owing to the small dimensions of the gut. Consequently, this appears to be the first record of an *Eimeria* from a cold-blooded animal discharging undeveloped oocysts.

Pérard (1925) has demonstrated that the failure of the oocysts to segment in warm-blooded animals is not due to the high temperature of the body, but their development on leaving the host is probably due to the indirect action of oxygen which inhibits the multiplication of the anaerobic intestinal bacteria. There is, therefore, no necessity to attribute the segmentation of the oocysts within the cold-blooded host to low temperature. *E. raiarum* thus provides the first exception to a rule originally based upon an erroneous hypothesis.

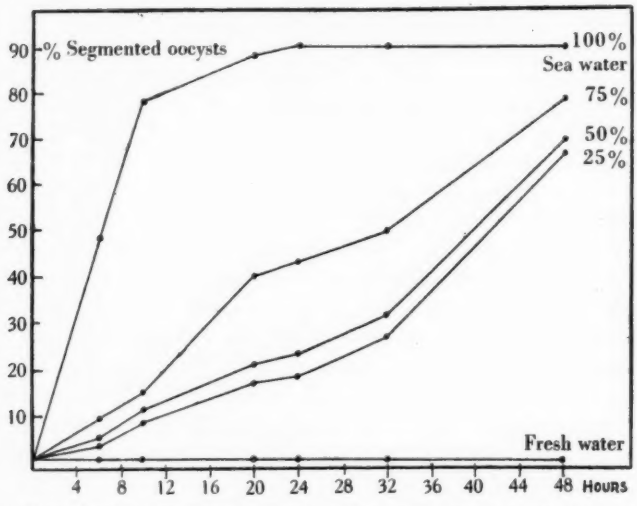
Very few observers have attempted to investigate the mechanism of the segmentation of oocysts. So far only four conditions were considered under which sporulation takes place, viz. (1) temperature, the importance of which has considerably diminished at present; (2) humidity, the role of which in the case of coccidia from aquatic animals is very doubtful; (3) aeration; and (4) fermentations. It is also known that the survival and segmentation of the oocysts is facilitated in 1–2 per cent solutions of chromic acid. The latter has a deleterious effect upon the microbes which inhibit the development of the oocysts, probably through the toxic action of their products of metabolism. The majority of observers using this technique appear to forget that it merely represents an artificial method directed against a factor inhibiting sporulation, but does not serve to elucidate the natural conditions under which this process takes place.

The unsegmented oocysts of *E. raiarum* provided excellent material for the study of this problem.

My attention was first directed to the well-known fact that the cytoplasm of the zygote becomes condensed before segmentation is initiated. As is shown in Pl. I, fig. 2, in immature oocysts the zygote fills almost the entire cavity; while fig. 3 in Pl. I shows one oocyst in the stage immediately preceding segmentation, the zygote having undergone the maximum condensation. Oocysts left in the intestinal mucus or placed in fresh water remain unsegmented for an indefinite period of time (Pl. I, fig. 2), whereas in sea water the zygotes become condensed sometimes within 1 hour, nearly all having undergone segmentation after 10 hours. As in the case of coccidioses in warm-

blooded animals, failure of the oocysts to segment in the mucus might be attributed to lack of aeration, if it were not for the fact that the behaviour of the oocysts differed so markedly in fresh and sea water, though both fluids were similarly aerated in the course of my experiments.

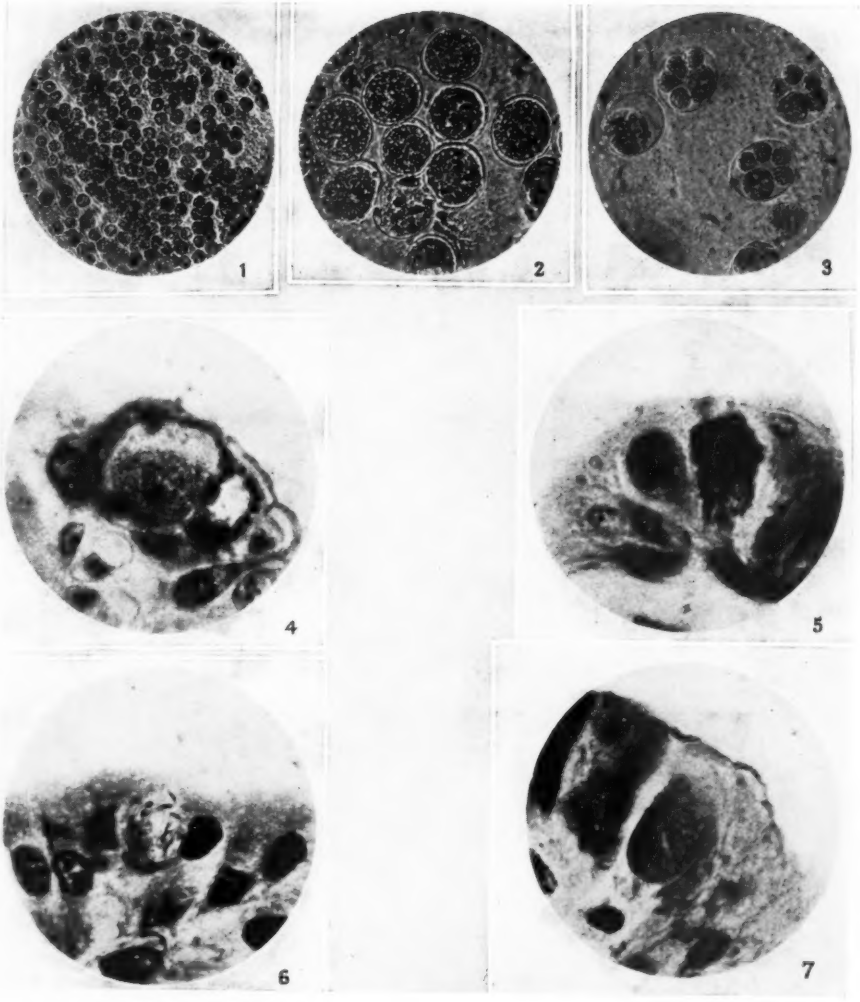
When the oocysts which had remained unsegmented for 8 days in the mucus and for 6 days in fresh water were transferred to sea water, 80 per cent of them became segmented within 12 hours. Thus, in both these cases the zygotes retained their viability and were capable of segmenting normally. The rapid condensation of the cytoplasm of the zygote in contact with sea water can only be explained by plasmolysis evoked by a hypertonic solution. In a recent paper on rabbit coccidia Cheyssin (1935) notes that the outer



Text-fig. 1. Chart showing the rate and percentage of segmentation of oocysts of *Eimeria raiarum* sp.n. in media with increasing concentrations of sea water.

membrane of the oocyst is permeable while the inner one is semi-permeable. When placed in a hypertonic solution the oocyst undergoes plasmolysis which, in the case of the rabbit coccidium, causes invagination of the membranes. When transferred into distilled water these oocysts are deplasmolysed.

In the case of the oocysts of *E. raiarum* an analogous phenomenon is produced, this time under natural conditions and without deformation of the membranes: sea water, a highly hypertonic solution, causes plasmolysis of the contents of the oocyst, viz. condensation of the cytoplasm of the zygote, which precedes its segmentation. In this connexion, observations were made upon the rate and percentage of segmentation of the oocysts in increasing concentrations of sea water. The results are given in the accompanying chart (Text-fig. 1) showing the correlation of these elements for a similar number of



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oocysts kept in pure fresh water, in 25, 50 and 75 per cent sea water diluted with fresh water, and finally in pure sea water. After 10 hours segmentation took place in 80 per cent of oocysts in pure sea water, and only in 15 per cent of those in 75 per cent sea water. After 24 hours 90 per cent of the oocysts segmented in pure sea water, 44 per cent in 75 per cent solution, 23 per cent in 50 per cent solution, 20 per cent in 25 per cent solution, and only 2 per cent in pure fresh water. After 48 hours the percentages in all dilutions were more or less equalized. These experiments indicate that the concentration of sea water is the most important factor governing the segmentation of the oocyst of *E. raiarum*. It is conceivable that certain constituents of sea water have a specific effect, but at the present stage of our knowledge it is more rational to assume that this process is due to simple osmotic phenomena. However, this interpretation does not necessarily apply to other coccidia: it has already been demonstrated that previous attempts to attribute the mechanism of segmentation of the oocysts to a single factor, viz. temperature, failed.

It is, therefore, possible that the conclusions arrived at in the present experiments are applicable solely to *E. raiarum*, a coccidium of a cold-blooded animal, the oocysts of which sporulate only in the external medium.

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EXPLANATION OF PLATE I

Fig. 1. Unsegmented oocysts of *Eimeria raiarum* sp.n. in intestinal mucus (× 100).

Fig. 2. Unsegmented oocysts kept 6 days in fresh water, with zygote filling the entire cavity (× 500).

Fig. 3. Oocyst with condensed zygote (plasmolysed) on the left, and three segmented oocysts (showing four sporoblasts and a residual body), 12 hours after transfer to sea water (× 500).

Fig. 4. Macrogamete (or early zygote) and a young female gametocyte with globular peripheral inclusions.

Fig. 5. Macrogamete and a young microgametocyte containing numerous nuclei.

Fig. 6. Fully developed microgametes.

Fig. 7. Late macrogamete or early zygote, possibly with a thin oocyst wall.

(*MS. received for publication* 14. III. 1936—Ed.)

STUDIES OF THE EFFECT ON *T. GAMBIENSE* AND *T. RHODESIENSE* OF PROLONGED MAINTENANCE IN MAMMALS OTHER THAN MAN; WITH SPECIAL REFERENCE TO THE POWER OF THESE TRYPANOSOMES TO INFECT MAN

V. THE EFFECT OF PROLONGED MAINTENANCE AWAY FROM MAN ON THE INFECTIVITY OF *T. RHODESIENSE* FOR MAN

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In this paper the history is given of the maintenance away from man of certain strains of *T. rhodesiense* originally isolated from man in Tanganyika Territory.

Information about the survival of *T. rhodesiense* in the African big game is of importance, even when tests on man are not included in the examination. The earlier investigations of these strains (Duke, 1935, 1935 *a*) supplied some facts about the survival of this species of trypanosome in game, and the history of some of the animals is now brought up-to-date to cover a period of some three years.

It is reasonable to assume that any wild animal wholly or partly diurnal in its habits that lives in game-tsetse country is either relatively inaccessible to Glossinae or is possessed of a high degree of tolerance to the trypanosomes they carry.

All the common large mammals that inhabit game-tsetse country are readily accessible to the fly during the day, even if, like the big carnivores, they are mainly active during the night. Anyone familiar with the African big game in its natural surroundings knows, for example, that lions and hyaenas roam about during the day, and that these animals, and even the leopard, commonly lie up for their siesta under trees or thickets rather than concealed in the traditional den. Lions especially are unprotected by their coat from biting flies, and I have seen a full-grown male asleep on its back in the bush in Uganda with its abdomen black with tiny *Lyperosia*.

It is therefore surprising to find that animals like the serval cat and the jackal, both of which are generally to be found in tsetse country, are extremely sensitive to the trypanosomes of the *brucei* group (Duke, 1933). It is true that the individuals of these two species hitherto tested came from tsetse-free areas, and it is possible that specimens from fly areas would be more resistant. On the other hand, it has been found that bushbuck and reedbuck and to a

less degree oribi, captured in fly-free areas and well accustomed to captivity before their exposure, suffered little or nothing from infection with *T. rhodesiense*.

No doubt much depends upon the condition of the animal. The part played by condition is shown by the way that individuals of the "immune" breed of dwarf cattle on the west coast, when fatigued by excessive exercise, succumb to a trypanosome infection which they had previously tolerated without apparent inconvenience. Similar effects were observed in East Africa when tamed zebra were employed as draught animals.

The jackals used at the Institute had not become accustomed to life in captivity at the time of their infection, but the serval cat had settled down and was in excellent condition when exposed to flies at the laboratory. Carmichael (1934) found the Uganda jackal very sensitive to *T. brucei*, and his animals were tame and in excellent condition.

Among other observations presented below it will be seen that the hyaena, an animal common in fly country, may serve as a reservoir for *T. rhodesiense* for at least 18 months. This is the more remarkable as both dogs and cats are exceedingly sensitive to the effects of this trypanosome. Moreover, neither this hyaena nor presumably its forbears had had previous contact with trypanosomes and certainly not with *T. rhodesiense*. Its tolerance therefore is natural to the species, and in striking contrast to the susceptibility of the domestic dog and cat and the jackal.

For an animal to serve as reservoir for a human trypanosome it must be able to tolerate the parasite without itself suffering serious impairment of health, and without damaging the power of the trypanosome to develop cyclically in tsetse and to infect man.

The importance of this last character has been revealed during recent investigations. Infectivity to man is, it is true, only one of the possible physiological attributes of trypanosomes. To man himself it is, however, extremely important; so much so, that this character has actually served as a basis of classification, although variation in regard to other hosts attracts little or no notice. There is good reason to believe that, as a criterion of species, it is unreliable, and that variations among individual strains and individual human beings play a large part in determining infection in nature (Duke, 1935 *a*).

Explanation of the schedules

Five strains are dealt with. Three of them, Tinde I, II, and III, have formed the subject of previous communications (Duke, 1935, 1935 *a*), to which reference can be made for a complete history. The other two, KB and KH, were received from Dr Corson in Tanganyika at the end of 1934.

The length and intricacy of some of the schedules given below are, it is hoped, justified by the existing scarcity of information in the literature about the infectivity of trypanosomes to man.

It is to be noted that where in the schedules a volunteer is shown as failing to become infected, this failure invariably coincided with the simultaneous infection of one or more control animals from the same inoculum; and where the failure followed the application of cyclically infected flies, those flies did actually bite the volunteer.

The conventional signs employed in previous publications from this laboratory are used again here. A thick line between passages means a cyclical passage by tsetse, a thin line an inoculation by the syringe. All animals shown in the schedule were infected on the dates accompanying their numbers. In the case of man, (+)=infection, (−)=non-infection. Where two or three animals or men carry the same date, they were all injected with the same inoculum. The only species of tsetse used was *G. palpalis*. An "infective" fly is one with infected salivary glands. An "infected" fly is not necessarily infective. m=monkey; p=guinea-pig.

An asterisk accompanied by a number and placed underneath the designation of a volunteer means that 6 c.c. of the volunteer's blood were inoculated into a clean *Cercopithecus* monkey that particular number of days after his exposure—to detect a possible cryptic infection in the volunteer. An infection of this kind did actually occur with strain Tinde II (e.g. volunteer O, strain Tinde II).

In recording the results of the several tests on the transmissibility of various trypanosomes in this paper, the headings of the test tables will be given for the first test only and not repeated for each test. For the method adopted in calculating the transmissibility index see Duke (1928).

Strain Tinde I. Isolated from a patient on 28. ii. 32. The laboratory history of this strain is shown elsewhere (Duke, 1935, 1935 *a*). The animals of interest here are: reedbuck I, infected by fly on 28. v. 32; oribi I, infected by fly from reedbuck I on 13. x. 32; reedbuck II and III; oribi II, infected on 29. iii. 33; and bushbuck II, infected by fly from reedbuck I on 13. x. 32. Of these, bushbuck II—a powerful ram in fine condition and during his captivity the father of two hybrids out of a situtunga doe—reedbuck II and III were alive at the time of writing.

From July to December 1933, nine volunteers were exposed to *G. palpalis* cyclically infective with this strain, but none of these men became infected. No inoculations of the blood forms of this strain were at that time made into man.

Reedbuck I. First infected on 28. v. 32. On the following dates 6 c.c. or more of citrated blood of this antelope were inoculated into clean monkeys: 8. iii. 32 (twice), 17. v. 33, 18. ix. 33, 17. xi. 33, 3. i. 34, 23. i. 34, 26. ii. 34, 4. x. 34, and 17. x. 34 (twice). None of these monkeys became infected. The animal had apparently recovered from its infection. On 17. x. 34 it died and the autopsy by Mr R. J. Mettam revealed that death was due to trauma.

Bushbuck II. First infected on 13. x. 32. The following inoculations of this antelope's blood were made into clean monkeys, the result being shown by

the + or − sign: 31. iii. 33 (+), 4. ix. 33 (−), 1. xii. 33 (−), 14. i. 35 (+) (m 1347), 13. ii. 35 (−), 19. ii. 35 (+) (m 1384), 18. vii. 35 (−), 24. vii. 35 (−), 7. viii. 35 (−). From this series of inoculations it was concluded that the antelope had gradually recovered from its infection.

The transmissibility of the trypanosome recovered from bushbuck II by inoculation of its blood into monkeys 1347, 1384 and 1402 (this last subinoculated from 1384) and also in the antelope itself before reinfection, was examined with the following results. Unfortunately it was not practicable to test the infectivity of the two gland-infected flies to man.

Infected animal	Period covered by test	Flies dissected			Flies living 25 days or more		Transmissibility index
		Total	No. infected	% infected	With infected glands	Total	
m 1347	25. i–10. ii. 35	507	14	2·76	1	11	0·2
Bushbuck II	9–29. iii. 35	520	4	0·77	0	4	0
m 1384	2–17. iv. 35	447	12	2·7	1	8	0·3
m 1402	18–26. iv. 35	220	1	0·45	0	1	0

The strain in this bushbuck was thus still transmissible by tsetse $2\frac{1}{3}$ years after its first introduction into the animal. It is noteworthy that both the gland infections were very light in character although the flies were 40 and 51 days old respectively, an observation of interest because the gland infections produced by strain Tinde I have always been of this unusual kind.

On 7. v. 35 two volunteers each received 1 c.c. of citrated blood of monkey 1402, and in 6 and 7 days respectively these men showed trypanosomes in their blood. In view of the results of the earlier tests on man this was quite unexpected, and in seeking an explanation the possibility had to be considered of an accidental infection of bushbuck II, in the kraal, from another antelope carrying either strain Tinde II or III, both of which are pathogenic to man. This contingency is, however, extremely improbable, for since March 1934 the two surviving bushbuck, Nos. II and III, have been isolated from the rest of the antelope in their own stable and run. Direct infection by *Stomoxys* is in these circumstances very unlikely. Moreover, between 14. i. 35 and 9. ix. 35 seven inoculations of bushbuck III's blood were made into clean monkeys, none of which became infected. The animal having thus for a long time shown no signs of trypanosomiasis, it could hardly have infected its stable companion.

There remain two possibilities: either the trypanosome during its residence in bushbuck II has reacquired the power of infecting man, or the forms in the monkey's blood were infective to man, whereas the metacyclic forms tested in the earlier investigation were not. As already stated, all the nine volunteers originally tested with strain Tinde I (Duke, 1935, 1935 *a*) were exposed to the metacyclic and not to the blood forms of the trypanosome. During the earlier investigations of the antelope reservoir, on two occasions and with two different strains of *T. rhodesiense*, flies infected from an antelope failed to infect man whereas flies infected from monkeys that had been inoculated

from the antelope did infect him. It has been suggested elsewhere that residence in primate blood may strengthen the pathogenicity of the trypanosome for man. Whatever be the explanation, the facts are clear: the blood forms of the trypanosome isolated from bushbuck II 859 days after the first infection of this antelope were, after 28 days in the blood of a monkey, infective to man.

On 8 and 9. viii. 35 bushbuck II was exposed to flies infective with *T. rhodesiense* (strain KB), and on 26 and 29. viii. 35 and again on 25. ix. 35, 1 c.c. of its blood infected clean monkeys (Nos. 1520, 1521, and 1553).

On 5 and 19. xi. 35 6 c.c. of the antelope's blood failed to infect clean monkeys; and between 22. x. 35 and 1. xi. 35, 374 clean laboratory-bred *G. palpalis* were fed twice on the bushbuck and not a single fly became infected.

These observations indicate strongly that this bushbuck, after a brief reinfection, has again thrown off the trypanosome. It is now about 3 years old and is an altogether more powerful and mature animal than its companion of the same age, bushbuck III.

Reedbuck II. The history of the infection of this animal is given elsewhere (Duke, 1934). Trypanosomes were first found in its blood on 24. viii. 33. On each of the following dates, 20. iii. 35, 15. iv. 35 and 12. viii. 35, 6 c.c. of its blood were inoculated into clean monkeys, no infection resulting. On 19 and 20. viii. 35 the antelope was bitten by two gland-infected flies carrying *T. rhodesiense* (strain KB), and on 4. ix. 35 and on 8. xi. 35, 1 c.c. of its blood infected clean monkeys (Nos. 1524 and 1570). Here again reinfection with another strain 2 years after the first infection took place promptly. A transmissibility test on monkey 1570 ran as follows:

m 1570	21. xi–3. xii. 35	475	26	5·4	9	10	4·8[1]

Five the of nine gland-infected flies of this test infected one volunteer, the incubation period being 7 days.

Finally, on 10 and 13. xii. 35, 2 c.c. of this reedbuck's blood failed to infect clean monkeys, the reinfection apparently having by then died out.

Reedbuck III. Originally infected on 3. vii. 33 by fly. A transmissibility test resulted as follows:

Reedbuck III	18–28. xi. 33	440	16	3·6	5	16	1·1

Inoculations of 6 c.c. of the reedbuck's blood were made into monkeys on 4. ix. 33, 19. iii. 35, 24. iv. 35, 30. iv. 35 and on 10 and 12. viii. 35, none of the animals becoming infected. Like reedbuck I, this antelope threw off its infection with strain Tinde I.

On 9. ix. 35 reedbuck III was bitten by two gland-infected flies carrying *T. rhodesiense* (strain Tinde II), and on 24. ix. 35 and 8. xi. 35, 1 c.c. of the reedbuck's blood infected clean monkeys (Nos. 1552 and 1571), showing that the antelope was not protected against reinfection with another strain of *T. rhodesiense.*

[1] Headings in this and all subsequent tables as in table on page 15.

The two flies that reinfected reedbuck III obtained their trypanosomes from a monkey that had itself been infected by an inoculation of human blood. Transmissibility tests were performed on the reedbuck and on monkey 1571, with the following results:

Reedbuck III	21. x–4. xi. 35	366	6	1·6	4	5	1·2
m 1571	21. xi–3. xii. 35	302	9	2·9	4	7	1·6

One of the four gland-infected flies of the test on monkey 1571 infected a volunteer, the incubation period being 10 days.

Finally, on 10 and 13. xii. 35, 2 c.c. of the blood of this reedbuck were inoculated into clean monkeys (Nos. 1591, 1595), and both monkeys became infected after an incubation period of 7 days.

Thus, with reedbuck III reinfection took place promptly and trypanosomes were still present in the peripheral blood 3 months later. The trypanosomes in a monkey infected from this reedbuck after reinfection were infective to man.

Oribi I. Infected 13. x. 32. Transmissibility tests on the trypanosome in this antelope were performed as follows:

Oribi I	9–26. iii. 33	403	6	1·5	1	5	0·3
Oribi I	28. xi–8. xii. 33	395	2	0·5	0	0	0

On 2. x. 33 10 c.c. of the animal's blood failed to infect a clean monkey. This oribi remained in excellent health until its death from undetermined causes on 5. xii. 34. An inoculation immediately after death of 10 c.c. of its blood into a clean monkey failed to produce infection.

Oribi II. Infected 29. iii. 33. A single transmissibility test was carried out on this antelope:

Oribi II	11–18. vii. 33	430	4	0·9	0	2	0

Inoculations of this oribi's blood were made into clean monkeys on 27. ix. 33, 1. xii. 33 and 6. iv. 35, none of the monkeys being infected. At the time of writing this oribi is alive and well.

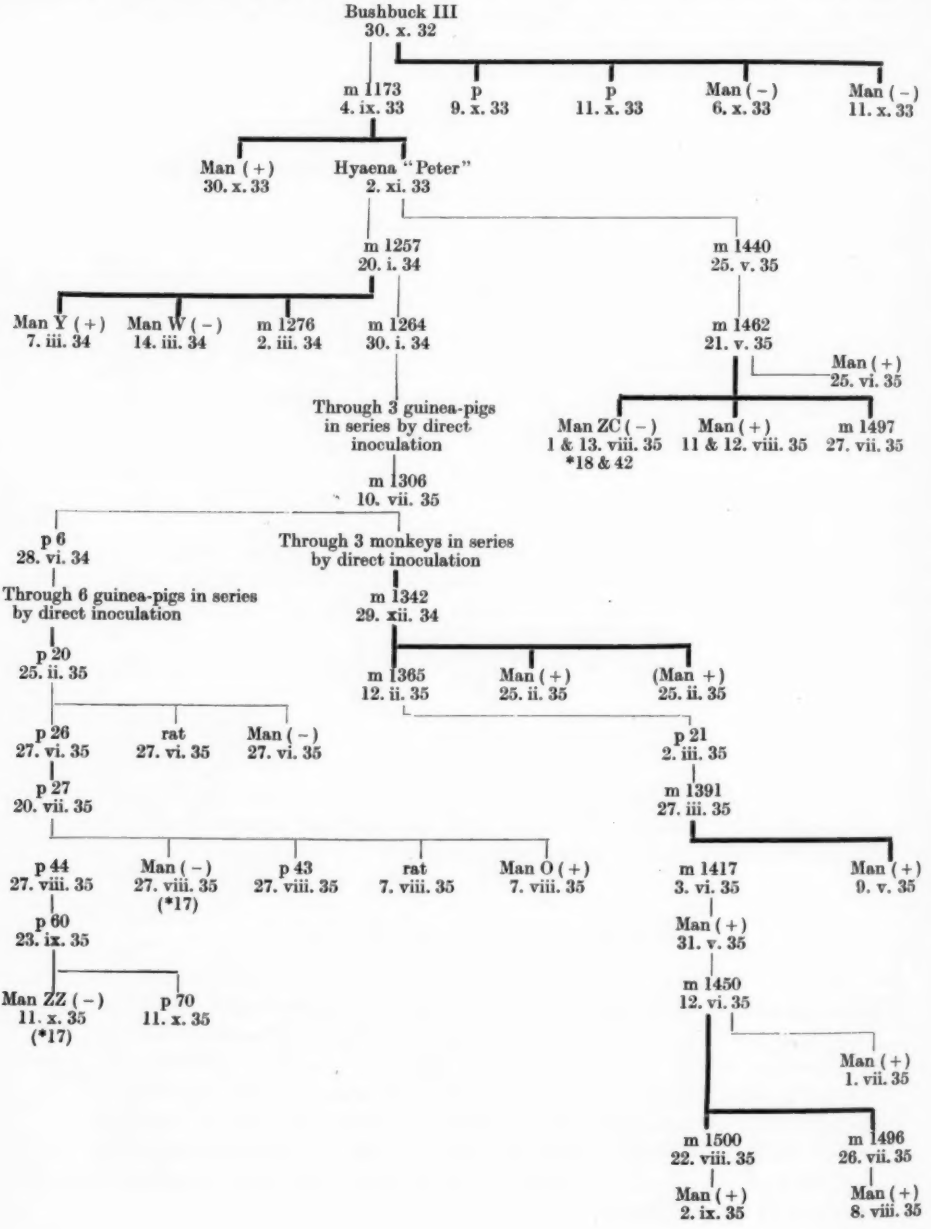
Summary of observations on strain Tinde I

(1) The trypanosome in the blood of bushbuck II was still transmissible by *G. palpalis* 2⅓ years after the animal's original infection with this strain. Trypanosomes from the blood of this antelope, isolated in a monkey, 859 days after its first infection, were found on inoculation into man to be still infective.

(2) In several of the other animals examined infection with strain Tinde I died out after a few months. Reinfection occurred readily, and the trypanosomes introduced by the reinfection retained their infectivity for man. In one antelope the reinfection persisted for at least 3 months; in another it appeared to die out rapidly.

Maintenance schedule of strain Tinde II

From patient on 7. iii. 32 into a white rat; thence through a second rat into a monkey and from the monkey by tsetse into a ntalaganya antelope on 26. vi. 32; thence by tsetse into bushbuck III on 30. x. 32.



Strain Tinde II

The top part of the accompanying schedule has already been produced elsewhere and fully discussed (Duke, 1935). The points now deserving attention are:

(1) Bushbuck III.

(2) Hyaena "Peter".

(3) The guinea-pig line of the strain, shown on the left-hand side of the schedule.

Note. The opportunity is taken here to correct certain errors overlooked during the proof correction of a previous paper on these strains (Duke, 1935 *a*).

(1) On p. 72 line 14; for "antelope" read "monkey".

(2) In the schedule for strain Tinde II on p. 74, the flies infected from bushbuck III were actually fed on two men, H and M (and not, as was shown, on one only), and also on two guinea-pigs. Both the guinea-pigs but *neither of the volunteers* became infected, as is correctly shown in Table II on the previous page. The second volunteer, M, in addition to being fed upon by five gland-infected flies, received subcutaneously the heavily infected glands of one fly.

(3) In the maintenance schedule of strain Tinde III on p. 77 the date of the inoculation of monkey 1140 from bushbuck I was 31. v. 33, not 26. ix. 33; also, m. 1157 was infected from m. 1154 by the syringe and not, as is shown, by fly.

Bushbuck III. First infected on 30. x. 32. Flies infected from the bushbuck in September 1933 infected monkeys and failed to infect man; but flies infected from monkeys inoculated with the blood of the bushbuck infected two volunteers (Duke, 1935 *a*).

This bushbuck is still alive (December 1935) and in excellent condition. The following inoculations were made of its blood (5–6 c.c.) into clean monkeys: 4. ix. 33 (m 1173) (+), 14. i. 35 (−), 14. ii. 35 (−). On 9 and 10. iii. 35 the animal was bitten by a single fly infective with *T. rhodesiense* (strain KB), this fly having previously infected two volunteers and several clean animals. After this exposure the following "negative" inoculations of the antelope's blood were made into monkeys: 1. iv. 35, 24. iv. 35, 14. v. 35, 31. vii. 35. Evidently this attempt at reinfection failed. On 29 and 30. viii. 35 the bushbuck was bitten by three gland-infected flies, two carrying strain KB and one strain Tinde II. On 9 and 12. ix. 35, 1 c.c. of its blood infected clean monkeys (Nos. 1528 and 1533) with *T. rhodesiense*. Thus bushbuck III recovered from its first infection, resisted reinfection 26 months later, and was reinfected by fly after a further 6 months.

On 5. xi. 35, 1 c.c. of the bushbuck's blood infected a clean monkey (No. 1569) after an incubation period of 7 days. Transmissibility tests on the trypanosomes in monkeys 1533 and 1569 were carried out together with tests on man:

m 1533	14–22. ix. 35	209	4	1·9	2	2	1·9
m 1569	14–24. xi. 35	278	10	3·6	2	4	1·8

One of the two gland-infected flies in the test on monkey 1533 fed on and failed to infect a volunteer; and both the infective flies in the second test fed on another volunteer without infecting him. All three of these flies when dissected were full of human blood.

Finally, on 10 and 13. xii. 35, 2 c.c. of the bushbuck's blood were inoculated into two clean monkeys, neither of which became infected. This antelope, like reedbuck III above, readily became reinfected, the trypanosomes persisting in the blood for some 2 months and then apparently again disappeared. Here, however, flies infected with the trypanosome after reinfection failed to infect man.

Hyaena "Peter". First infected 2. xi. 33. Arrived at the Institute as puppy from a fly-free area. Thoroughly domesticated, good tempered and in first-class condition. Unfortunately, owing to its strength and a growing reluctance to submit to forcible manipulations, it is becoming very difficult to handle. As, however, the animal was recently shown to be still infected there is no call for superinfection, with all the difficulties attendant on the employment of tsetse.

The behaviour of the strain on the two occasions when it was isolated from the hyaena is shown in the schedule. It will be seen that twelve different volunteers were tested and all save two, W and ZC, became infected. Of these two men, W has been dealt with elsewhere (Duke, 1935). ZC had been infected previously on 13. iv. 34 with strain Br (Duke, 1935 *a*), receiving his last dose of "Bayer 205" on 6. v. 34. It has been shown elsewhere that any immunity possibly conferred by this first infection would disappear in 6 months or so (Duke, 1936). On 10. vi. 35 ZC received an inoculation of blood containing *T. gambiense* (strain KDY) and did not become infected. Volunteer ZC may therefore be regarded as unusually resistant to trypanosomes—in all probability a natural resistance.

Turning now to the guinea-pig line of this strain shown on the left of the schedule, there are distinct signs of impairment of pathogenicity to man. Of four volunteers exposed, three failed to become infected.

Volunteer O's infection is of interest as an example of an infection of man by a trypanosome barely able to establish itself. This man has been referred to elsewhere (Duke, 1936). The details are as follows: 20 months previously O had been experimentally infected with *T. rhodesiense* and promptly treated and cured, the last dose of "Bayer 205" being administered on 6. xii. 33. On 7. viii. 35 he and a white rat received respectively 1 c.c. and $\frac{1}{4}$ c.c. of the citrated blood of guinea-pig 27 of the schedule. On 29. viii. 35, 5 c.c. of O's blood were inoculated into a clean monkey, and trypanosomes appeared for the first time in the course of daily examinations of thick stained films of this monkey's blood on 19. ix. 35. All this time O professed himself to be in perfect health. On 9. ix. 35, before a diagnosis had been made, he was reinoculated with the same strain of *T. rhodesiense*, this time from a monkey. On 18. ix. 35 his blood again infected a clean monkey, this time after an incubation

period of 9 days. Thick stained films of the man's blood taken and examined on 23, 24, 25, 26. ix. 35 were "negative", but on 27. ix. 35 scarce trypanosomes were seen for the first time and were thenceforth present each day until 3. x. 35, when treatment started. His temperature, taken twice daily, rose to just above normal for the first time on 3. x. 35, and he complained of no symptoms until that date. Treatment was immediately effective.

Of the three volunteers who failed to become infected with the guinea-pig line, ZZ may be regarded as possessing unusually high resistance against trypanosomes (cf. strain KB below). In the case of all the three men the control animals inoculated at the same time promptly became infected.

Situtunga II. This animal has been referred to in the first paper of this series as having resisted infection with *T. gambiense* on 2 and 3. iii. 35. Its subsequent history is as follows:

On 14. ix. 35 the antelope was bitten by an infective fly carrying *T. rhodesiense* (strain Tinde II). On 30. ix. 35, 4. x. 35 and 5. xi. 35, 1 c.c. of the animal's blood infected clean monkeys.

Early in December conjunctivitis appeared in both eyes, the upper and lower lids swelling considerably: the symptom disappeared gradually in the following 3 weeks. As there appeared to be no question of trauma this condition must, I think, be referred to the trypanosome infection, an opinion shared by Mr R. W. Mettam, Veterinary Pathologist, who commented upon the rarity of this symptom in his experience of trypanosomiasis in ruminants.

It would appear therefore that this situtunga, having resisted infection with *T. gambiense*, promptly reacted to *T. rhodesiense* in a manner most unusual in antelope. During the attack of conjunctivitis trypanosomes were not found in thick blood films of the animal's blood and on 13 and 23. xii. 35, 2 c.c. of its blood failed to infect clean monkeys.

Summary of results obtained with strain Tinde II

(1) A spotted hyaena (*Crocuta crocuta germinans* Matschie) supported an infection with *T. rhodesiense* for at least 22 months without suffering any apparent inconvenience.

(2) A subinoculation of the hyaena's blood was made 48 days after its first infection, and the monkey inoculated became infected. Some 6 weeks later flies infected from this monkey infected one (Y) and failed to infect a second volunteer (W). The strain was then split into two lines, one maintained by direct passage through a series of guinea-pigs and the other, the main line, passed through a series of monkeys, sometimes by fly, sometimes by direct passage.

Thirteen months after its isolation from the hyaena the main line was tested in fly on two volunteers, both becoming infected; and subsequently at intervals five more volunteers were exposed and all became infected.

(3) A second isolation of the trypanosomes from the hyaena was made 569 days after the first infection of the animal, again into a clean monkey.

When this monkey became infected a second monkey was inoculated from it, and 1 c.c. of the blood of this second monkey mixed with citrate infected a volunteer after an incubation period of 5 days.

Laboratory-bred *G. palpalis* infected from this second monkey infected one volunteer but failed with a second (ZC). This second man was in addition inoculated subcutaneously with the heavily infected gland of one of these flies, and the other gland was inoculated simultaneously into a white rat; the rat showed trypanosomes 6 days later, but the man did not become infected.

These observations add to the evidence already accumulated at this laboratory that sojourn in guinea-pigs tends to reduce the pathogenicity of a trypanosome for man. They also illustrate well the differences between different people in their resistance to trypanosomes.

(4) The contrast between the two lines of this strain of trypanosomes in their power of infecting man is interesting. The main fly-monkey line proved infective to all (eight) volunteers exposed to it, whether transmitted by *Glossina* or by the syringe: the guinea-pig line failed to infect two volunteers and infected a third.

(5) The schedule attests the complexity of the relations obtaining between the *brucei* group and man. In comparison with other strains of *T. rhodesiense* under investigation at this Institute, this strain earned the reputation of being strongly pathogenic to man.

(6) An adult situtunga ram which had resisted infection with *T. gambiense* was infected by fly with strain Tinde II.

Shortly after its infection the animal developed conjunctivitis in both eyes, the inflammation dying down in the course of 3 weeks—a very unusual reaction in antelope.

Note. In the description already published of the tests on man performed with strain Tinde II after passage through the hyaena, it was recorded (Duke, 1935 *a*) that of two volunteers, Y and W, both of whom were exposed to tsetse cyclically infected from monkey 1257, only Y became infected. There was at the time some doubt whether W really had been bitten. Work done by Dr K. Mellanby at this Institute during the last few months has, however, revealed some interesting facts about the rate of digestion of *G. palpalis* under laboratory conditions. Applying this knowledge to the records of the exposure of volunteer W, it is certain that two gland-infected flies bit this man. The two men were bitten by different flies, and it is possible that individual flies vary in their infectivity to man; a difference between the natural resistance of the two men is, however, a more probable explanation.

Strain Tinde III. The earlier history of this strain is given elsewhere. It was isolated from a patient on 21. ii. 32. After being 98 days in bushbuck I it was passed cyclically by fly to a fowl and thence by the syringe, via a monkey, into the first of series of oxen. The strain remained in oxen for 294 days. It was then inoculated by the syringe, via a white rat, into a monkey,

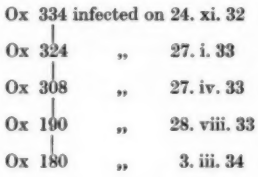
and from this monkey flies were infected. These flies bit and infected two volunteers (Duke, 1935 *a*).

Bushbuck I. Died on 29. x. 34, 860 days after its first infection with strain Tinde III. The earlier behaviour of the trypanosome in its blood has already been described (Duke, 1935 *a*).

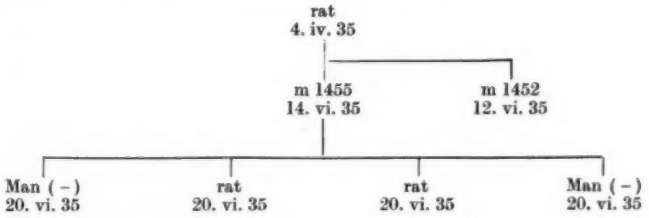
On 19, 26 and 29. x. 34, 10 c.c. of the animal's blood were inoculated into clean monkeys, none of which became infected. During the last few weeks of its life this bushbuck lost condition rapidly. The autopsy performed by Mr R. W. Mettam revealed no definite lesions, the report closing with the comment: "It is impossible to regard trypanosomiasis as the cause of death."

Another line of the same strain was started in a series of guinea-pigs, beginning with the cyclical infection of the first pig and continued through fourteen pigs by direct inoculation over a period of 18 months. It was then inoculated on two occasions into the same volunteer without infecting him (Duke, 1935).

The completed history of the ox line supplied by Mr R. W. Mettam is as follows, the date of ox 324 being now shown correctly.



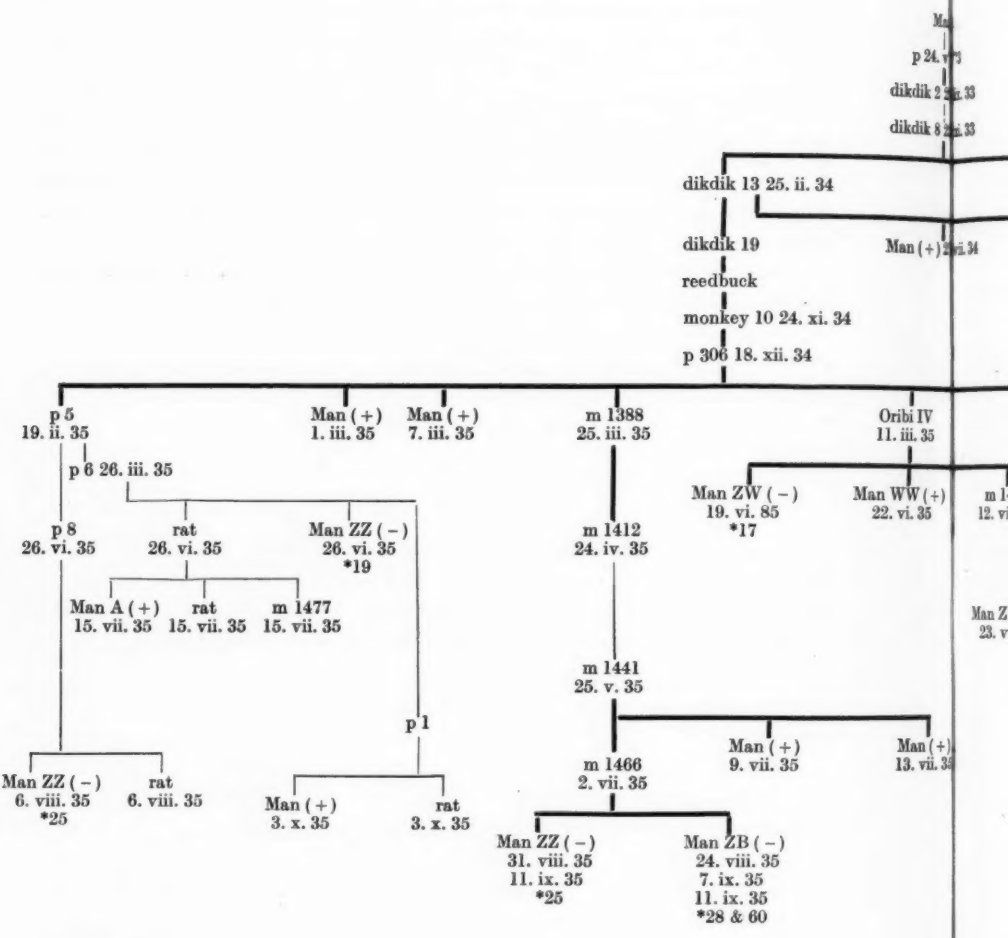
Ox 180 eventually recovered untreated, and was reinoculated on 11. xii. 34 with a derivative of the same strain that had been taken from ox 334 in December 1932 and maintained subsequently in a long series of laboratory rodents. From ox 180 a rat was inoculated and sent to the Institute.



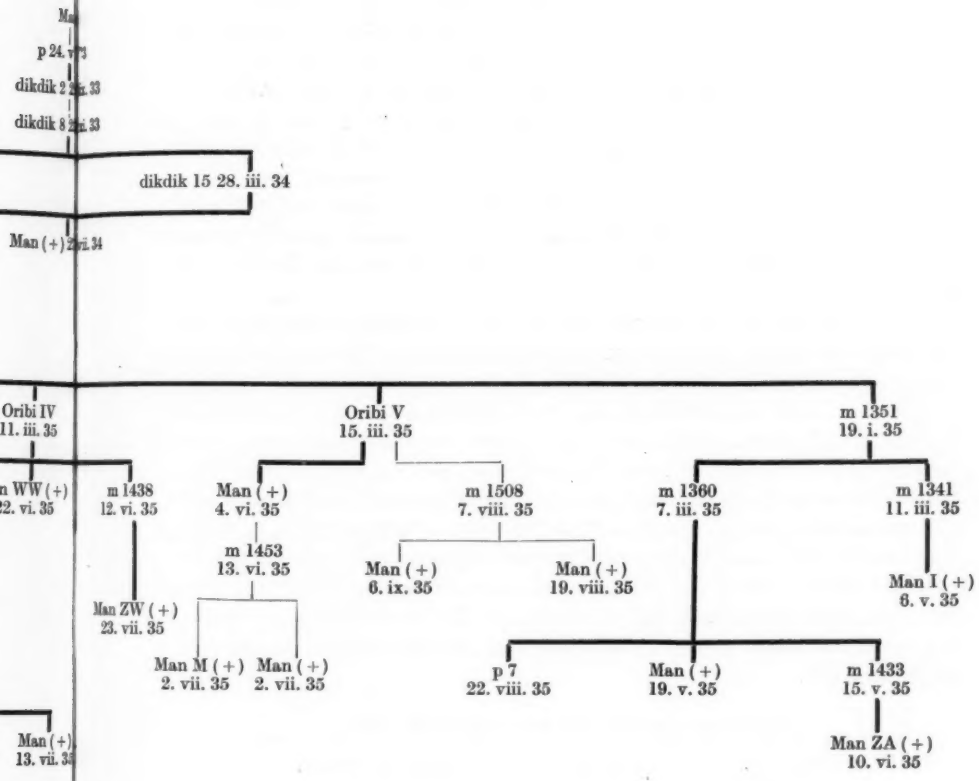
The disease in the two monkeys was extremely rapid, lasting 8 and 14 days respectively.

Yet another guinea-pig line of strain Tinde III was started on 28. iii. 34 and passed through eight pigs by direct inoculation. A ninth pig was infected on 26. viii. 35, and from this animal a volunteer and two control pigs were inoculated. Both controls were infected, the man was not.

Maintenance schedule of strain



ance scheme of strain KB



Summary of results obtained with strain Tinde III

The history of this strain prior to the present investigations showed that it was as a rule readily pathogenic to man during the first 18 months after its original isolation from the patient. Eight volunteers were infected.

On the other hand, four men failed to become infected with this trypanosome. Flies infected from bushbuck I failed to infect three volunteers out of four, whereas flies infected from a monkey inoculated with the bushbuck's blood infected all the men (six) exposed. An example of the difference in the susceptibility of two individual volunteers was supplied in these earlier experiments, the same flies infecting man K and failing to infect man L.

Bushbuck I before its death appeared to have thrown off its infection with *T. rhodesiense*, and its death cannot be ascribed to trypanosomiasis. It has been shown above that the ox line of the strain, when tested again in man some 15 months later, had lost its pathogenicity. A second guinea-pig line, started in March 1934, was tested 17 months later on man and found to be non-infective.

Strain KB. This strain was supplied by Dr J. F. Corson, and the earlier passages in the schedule on pp. 24 and 25 were carried out by him at Tinde. The strain reached Entebbe in guinea-pig 306.

Hybrid I. Born of a situtunga doe, by a bushbuck ram on 13. ix. 33. On 26 and 28. i. 35, 6 c.c. of this antelope's blood were inoculated into clean monkeys and no infection resulted. The animal up to this time had been kept, together with oribi V, in a separate pen in the antelope house, and not exposed to experimental infection. On 28. i. 35 it was bitten by a fly carrying *T. rhodesiense* (strain KB), infected from pig 306, and trypanosomes appeared in the antelope's blood on 4. ii. 35. Injections of 2 c.c. of the hybrid's blood were made into clean monkeys on 29. vii. 35, 30. ix. 35, 10. xii. 35 and 13. xii. 35, none of the monkeys becoming infected. The infection in this hybrid had rapidly died out.

Summary of results obtained with strain KB

The following points on the schedule call for special comment.

(1) *Volunteer ZZ.*

(*a*) Inoculated on 26. vi. 35 from pig 6; did not become infected: control +.

(*b*) Inoculated on 6. viii. 35 from pig 8; did not become infected: control +.

(*c*) On 31. viii. 35 was fed upon by two gland-infected flies carrying strain KB, and again on 11. ix. 35 by two more flies infective with the same strain. These flies failed to infect both ZZ and ZB: control +.

(*d*) On 11. x. 35 ZZ was inoculated from guinea-pig 60 carrying strain Tinde II (cf. *supra*); again he did not become infected: control +.

It is therefore probable that this man possessed an unusual degree of natural resistance against *T. rhodesiense*. As far as is known he had never before been exposed to infection and had certainly never received any trypanocidal drugs.

(2) *Volunteers ZW and WW.* ZW was bitten, on 18 and 19. vi. 35, by two flies infective with strain KB. One of these flies on 22. vi. 35 infected volunteer WW; ZW did not become infected. On 23. vii. 35 ZW was infected by the bite of a single fly (infected from monkey 1488), the incubation period in ZW being 16 days.

This apparently is another instance of a phenomenon described elsewhere (Duke, 1935 *a*); namely, a fly infected from an antelope failing to infect man, whereas a fly infected from a monkey that has been infected from that antelope succeeded.

These two men, ZW and WW, illustrate also the difference in the resistance of the two individual volunteers when exposed to the same risk: neither of them had been previously exposed to experimental infection or to trypanocidal drugs.

(3) *Oribi V.* This animal was conceived at the laboratory and born on 24. xi. 34. When weaned it was isolated from its companions, and its blood examined by the microscope and by subinoculation into clean monkeys, no trypanosomes being detected. On 11. iii. 35 it was exposed to flies infective with strain KB, and 8 days later trypanosomes were found in its blood. On 7. viii. 35, 1 c.c. of its blood infected a clean monkey. On 1. x. 35, 19. xi. 35 and 5. xii. 35, inoculations of 2 c.c. of its blood failed to infect monkeys. A transmissibility test on the antelope gave the following result:

Oribi V	26. iv–9. v. 35	294	6	2·0	4	4	2·0

Between 21. x. 35 and 4. xi. 35, 371 clean laboratory-bred *G. palpalis* were fed each fly twice on the antelope; none of the flies became infected.

This young oribi has therefore to all appearances thrown off its infection, the last evidence thereof being obtained some 5 months after the onset.

Oribi IV. Infected on 11. iii. 35. A transmissibility test on the antelope in April and May gave the following result:

Oribi IV	26. iv–6. v. 35	305	3	0·9	3	3	0·9

Note that with both these oribi the metacyclic index of the trypanosome is 1.

(4) It will be seen from the schedule that although still in general terms "pathogenic to man", this strain exhibits signs of weakness in this respect. Volunteers ZZ and ZB failed to become infected although intensively exposed, and volunteer ZW escaped one exposure. Fifteen other volunteers became infected at their first exposure.

Strain KH. Supplied by Dr Corson, arriving at Entebbe in guinea-pig 305 in December 1934. On 8. ii. 35 guinea-pig 4 was inoculated from guinea-pig 305; on 5. vi. 35 guinea-pig 3 from guinea-pig 4, and on 3. viii. 35 guinea-pig 2 from guinea-pig 3.

On 25. x. 35 the blood of guinea-pig 2 infected a clean volunteer after an incubation period of 9 days.

Discussion

The *Annual Report of the Veterinary Department of the Uganda Protectorate* for 1934 contains the following paragraph about antelope and trypanosomes (para. 52):

"It seems evident from the results of these experiments that in a tsetse area these animals pass through a transient infection with the prevalent trypanosome and thereafter resist further infection. They would, therefore, except for a comparatively short period of their lives, be non-infective to tsetse flies. Furthermore, this state would account for the fact that so few of the animals shot in areas swarming with tsetse fly are found to be harbouring trypanosomes, although the high percentage of infection in the south Ankole *G. morsitans* belt (5·5 per cent) is difficult of explanation if the foregoing hypothesis is correct and further information is required as to the age when 'game' is most susceptible to infection."

It is not possible at the time of writing to ascertain the data from which the percentage of infected *G. morsitans* mentioned in the *Veterinary Report* was calculated, nor the number of game animals examined. Actually, in comparison with published figures for other fly belts, 5·5 per cent is by no means high, if it relates to all three groups of pathogenic trypanosomes. It must be remembered, also, that the altitude of this fly belt is over 4000 ft. Much depends also on the method employed in diagnosing infection in the game.

The results presented in this paper do not justify the same conclusion for *T. rhodesiense*. It has been seen that this trypanosome may survive in antelope for more than 2 years in a form transmissible by tsetse, and this in an animal whose average life in captivity is 8 years (Flower, 1931) can hardly be described as a transient infection. A little later the trypanosome had, it is true, apparently disappeared from the blood, although this was not absolutely proved. Certainly reinfection—or, perhaps, superinfection—took place immediately the animal was again exposed to fly. And likewise in the other three antelopes exposed to reinfection.

Valenza (1935), working with *T. congolense* and guinea-pigs, found that reinfection of an animal recently sterilized by treatment sometimes resulted only in a rise of temperature without any invasion of the blood by the trypanosome. If a second reinfection was then carried out trypanosomes became established. Apparently the first reinfection had exhausted the circulating antibodies formed during the original infection, leaving the animal once again susceptible.

There is of course an enormous difference between experiments of this kind and the infections of antelope which have just been described. Nevertheless, it is well to bear in mind the possibility that an immunity acquired as the result of a trypanosome infection may be attenuated or neutralized by a succeeding abortive infection introduced during the period when that immunity is still active.

The investigations into the behaviour of *T. rhodesiense* in antelope carried out at this Institute during the last 3 years indicate that this trypanosome is very much better adapted to antelope than *T. gambiense*.

In the succeeding paper of this series (Duke, 1937) it is shown that *T. gambiense* in its ordinary East African form has difficulty, at any rate shortly after its isolation from man, in maintaining itself in antelope. Apparently *T. rhodesiense* experiences no such difficulty, and may persist for months or years in an antelope in a form transmissible by tsetse.

The adaptation of *T. rhodesiense* to antelope follows the lines already described in my earlier papers. Trypanosomes soon become very rare in the peripheral blood and do no apparent harm to the animal. Only a small percentage of the flies that feed on the antelope will become infected, but all infected flies will, if they live for 25 days or more, develop gland infections; that is to say, the metacyclic index of the strain is unity (Duke, 1928).

This is well illustrated by strain Tinde II in bushbuck III and by strain Tinde III in bushbuck I (cf. Duke, 1935 *a*, pp. 73 and 78). The three successive transmissibility tests on bushbuck I were as follows:

Bushbuck I	12–17. ix. 32	488	18	3·6	2	15	0·4
„	3–8. vii. 33	516	3	0·6	3	3	0·5
„	8–24. ii. 34	591	6	1·0	4	4	1·0

This state of affairs is, I believe, a sign of biological adjustment between the trypanosome and its host. According to experience acquired at Entebbe, with most strains, both of *T. gambiense* and *T. rhodesiense* maintained in laboratory animals and taken up by tsetse, a proportion—and sometimes a large proportion—of the infected flies develops gut infections which within 30, 40 or 50 days do not extend to the glands, although the cycle in other flies is completed in 20–25 days. Some strains, especially of *T. gambiense*, readily infect the alimentary canal of the fly but never the glands, and are accordingly only transmissible by the direct method (Duke, 1930).

Taylor (1932) was led by his investigations to believe that the establishment of flagellates in the proventriculus of the fly is the real criterion of transmissibility, and that once the proventriculus is infected invasion of the salivary glands is merely a matter of time. Van Hoof and Henrard (1934) have recently examined this problem, without, however, reaching any definite conclusion. In all save one of their strains the proventricular index is greater—often considerably greater—than the salivary gland index. Interest attaches especially to one strain in their report, "Onema". The proventricular and salivary gland indices of this strain were respectively 1·5 and 0, calculated for the 136 flies fed on the patient; whereas in 123 flies fed on guinea-pigs infected with this strain, four flies were found with the proventriculus infected and in one of them the glands also. The authors are inclined to regard this strain as illustrative of the greater value of the proventricular than the salivary gland index in determining the transmissibility of a strain. They admit,

however, that the number of tsetse employed in the test on the patient was inadequate for a final conclusion to be drawn that the strain in man was indeed incapable of infecting the glands of the fly, and this appears to me a reasonable explanation of the apparent discrepancy between the two sets of figures.

My belief that there exist strains which are still capable of infecting the intestine but not the glands of *Glossina* is based, among other facts, on the discovery in a number of different strains, of flies with heavy gut infections but no gland involvement, dissected 30, 40, 50 or 60 days after their infecting feed—flies taken from the same boxes as others in which the cycle was completed in from 20–25 days (Duke, 1933 *a*).

My own dissections have never included a systematic examination of the proventriculus of all infected flies. The immediate object was to determine whether or not the glands were invaded in infected flies of sufficient age for the cycle to have been completed—flies, that is, which had not had an opportunity of picking up a secondary infection from the animal they themselves had infected.

According to the evidence that has given rise to my views on the existence of non-transmissible strains the loss of the power to invade the glands is the first demonstrable change that occurs in a "degenerating" strain. Whether this change is associated with loss of the ability to develop in the proventriculus, I do not know. It is, however, certain that strains exist (for example, *T. gambiense*, strain XXIX (Duke, 1930)) that produce a relatively high percentage of infected flies in none of which are the glands infected. Unfortunately for the present discussion, as already stated, the proventriculus in the flies infected with strain XXIX was not examined.

I have maintained for years past that strains of trypanosomes possess characters of their own, and that one of the points wherein they differ is transmissibility by tsetse. Some strains when recovered from man or animals in nature are actually non-transmissible by *Glossina*. As long as a strain is unmixed it will retain its own characters: some strains, being more powerful than others, will preserve their qualities in spite of admixture. The possibility that strains of trypanosomes possess individuality is, however, at the moment largely ignored by some investigators. Variations in the host, definitive or intermediate; temperature; humidity; altitude, even—all exert a prior claim to attention in discussions on the epidemiology of human trypanosomiasis. Van Hoof and Henrard found their strains possessed of different and characteristic degrees of cyclical transmissibility by tsetse, resistance to arsenic and general pathogenicity; and they noted that a change in one character does not necessarily affect the others. Similarly with Taylor's strains in Nigeria, some were more, some less transmissible (Taylor, 1932).

While therefore established facts can wait until such time as the appeal of other lines of research in the field of trypanosomiasis is exhausted, it is of little use extending this dissertation, which assumes that different strains do

possess definite characters which respond in a definite way to their environment. The theory seems to fit the facts, and the facts the theory. The experimental conditions under which these facts have been established are remarkably constant, even if in some ways peculiar—*G. palpalis*, a mild and equable climate, an altitude of 3–4000 ft., and a fairly wide range of strains from Central and East Africa. The fact that investigators in other hotter parts of Africa have obtained higher percentages of infective flies in their experiments, the cycle also taking less time, suggests that climate does play a part, probably by its effect on the intermediate host. Van Hoof and Henrard decided that temperature, *per se*, did not affect the results of their transmission experiments. My own work and some recently published observations by Lester in Nigeria suggest strongly that in one and the same area different species of tsetse differ in their transmitting power (Lester, 1934).

But it is unlikely that the biological principles determining the behaviour of a trypanosome differ in different regions of Africa, and it is on the realization of these principles that ultimate success in controlling the disease depends. Van Hoof has recently informed me by letter that he has obtained results which show that non-transmissible strains do occur in man in the Belgian Congo. One of his strains, tested with some 1500 *G. palpalis*, behaved in a manner essentially similar to strain XXIX of the Entebbe series, a strain which was isolated from a European in Uganda. 4612 flies were dissected after feeding on different animals infected with strain XXIX: sixty-six of these developed massive gut infections, but in none were the salivary glands infected; 75 per cent of the infected flies lived 25 days or longer (Duke, 1930).

Those associated with the League of Nations Sleeping Sickness Commission will see in these investigations at Leopoldville an encouraging result of the endeavours made at that time to ensure coordination of research in various parts of Africa. A great deal of transmission work has been carried out by various investigators since that Commission was disbanded in 1927, and there have been opportunities of clearing up some unsolved and important biological problems, in addition to the attainment of the special objectives pursued by each investigator. It should not be difficult for those engaged on the experimental transmission of the human trypanosomes by tsetse to preserve and publish any figures bearing on these particular subjects, and if necessary even slightly to rearrange their scheme of research in order to secure such information. For among the outstanding problems of human trypanosomiasis there is surely none more important and worthy of study than the power of a trypanosome to utilize its insect vector, and the effect on this attribute of prolonged residence in its natural vertebrate hosts.

Conclusions

It is with some reluctance that an attempt will now be made to draw general conclusions from these observations. Only a few antelope have been studied and only a few strains. On some points the evidence is clear; on others, merely suggestive. It is satisfactory that the evidence obtained in the investigations of the last few years at this Institute has been on the whole confirmatory of previous inferences, and that certain ideas put forward tentatively in past publications now rest less on imagination and more on fact.

The closure of the Institute makes it, however, necessary to produce at this juncture some kind of a general summary.

(1) *T. rhodesiense* may survive in antelope for at least $2\frac{1}{3}$ years in a form transmissible by tsetse.

(2) Strains of *T. rhodesiense* differ in their adaptability to antelope.

(3) Individual antelope react differently to the same strain; some throw off infection more quickly than others. There is some evidence to show that of the four species examined, bushbuck and reedbuck are better suited to requirements of the trypanosome than oribi and situtunga.

(4) Strains of *T. rhodesiense* differ in their power to infect man; some lose this power more readily than others.

(5) There is reason to believe that with strains whose pathogenicity to man is neither very stable nor pronounced, the inoculation of the blood forms of the trypanosome is more likely to produce infection in man than the introduction of metacyclic forms by the bite of an infective tsetse.

(6) Some new evidence has been brought forward that flies infected from an antelope carrying *T. rhodesiense* may fail to infect man, whereas flies infected from a monkey that has been infected by an inoculation of the same antelope's blood may infect man.

(7) A study of all the results obtained during the last few years at Entebbe leads me to conclude that although an antelope may remain infected with *T. rhodesiense* for a year or two, there is a definite tendency for the trypanosome in these circumstances to lose its infectivity for man. An important point is this. Although a trypanosome after long residence in antelope, when isolated in laboratory animals and tested from time to time in man, may infect all or the majority of the volunteers employed, nevertheless when the tests are performed by means of flies infected from the antelope itself the results on man may be very irregular. This irregularity cannot be explained by the failure of the flies actually to bite the volunteer.

(8) After a time, varying according to the individual and species of animal from a few months to more than 2 years, *T. rhodesiense* appears to die out in the blood of antelope. Such an animal is, however, susceptible to reinfection with another strain of *T. rhodesiense*, a very short time after the disappearance

of the first infection. This suggests that immunity may gradually be established in an antelope repeatedly exposed to infection.

Whether an antelope ever becomes *permanently* immune against *all* strains of *T. rhodesiense* has not been determined, but it appears likely that this process will be slow.

(9) Interesting results have followed the testing on man of the trypanosome in the reinfected antelope. This has been done with one bushbuck and two reedbuck. In each test several flies with heavy gland infections were proved (by dissection and finding blood corpuscles) to have bitten clean volunteers. The reinfection strain in the bushbuck failed to infect man; those of the two reedbuck succeeded.

It was found some years ago with sheep and goats that a prolonged infection with *T. gambiense* resulted in the reduction or complete loss of the transmissibility of the strain in that animal. If, after this had happened the animal was then superinfected with another and readily transmissible strain, and the trypanosomes in the superinfected animal were examined one month or so later, their transmissibility by tsetse was either much reduced or lost.

The reinfection experiment with bushbuck III reveals similar features. The transmissibility of the reinfected strains was, it is true, unimpaired—we know now that *T. rhodesiense* is much better adapted to life in antelope than *T. gambiense*—but the partially immunized animal in this case has apparently exerted an effect on another physiological quality of the trypanosome, viz. its pathogenicity to man, as tested in two volunteers.

(10) A spotted hyaena (*Crocuta crocuta germinans* Matschie) was infected with *T. rhodesiense*. Two years later the animal appeared to be in excellent health, never having manifested any symptoms of illness. 569 days after its first infection trypanosomes were isolated from this hyaena and found on frequent tests to be regularly pathogenic to man.

(11) Prolonged maintenance of *T. rhodesiense* in guinea-pigs has been found on several different occasions to lead to impairment of the pathogenicity of the strain for man.

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(*MS. received for publication* 10. III. 1936.—Ed.)

STUDIES OF THE EFFECT ON *T. GAMBIENSE* AND *T. RHODESIENSE* OF PROLONGED MAINTENANCE IN MAMMALS OTHER THAN MAN; WITH SPECIAL REFERENCE TO THE POWER OF THESE TRYPANOSOMES TO INFECT MAN

VI. STRAIN "KAHONDERA" FROM SOUTHERN RHODESIA, AND CONCLUDING OBSERVATIONS ON THIS SERIES OF PAPERS

By H. LYNDHURST DUKE

From the Human Trypanosomiasis Research Institute, Entebbe, Uganda

In this, the last paper to be published from the Uganda Institute, after an account of the strain that forms its main subject, the origin of *T. rhodesiense* will be briefly discussed in the light of the investigations recorded in this series of papers and a few general observations will be made.

The Kahondera strain, the subject of this paper, was isolated from a native of Gowe in the Hartley-Sebungwe area of Southern Rhodesia. The strain has been studied by Bevan, who kindly sent it to Entebbe by air in two white mice.

Kahondera was one of three infected natives detected by Dr Blair during an exhaustive survey of the area, one of the other cases being Kahondera's son. The Gowe settlement coincided with one of certain small areas depicted on the tsetse maps of the Protectorate and called by Jack "residual foci", where tsetse survived the great rinderpest outbreak of 1896.

The conditions at Gowe were therefore peculiarly favourable to the development of a human strain of *T. brucei*, for man had there been in contact with the fly and trypanosome for many years.

In his paper on the origin of human trypanosomiasis in Rhodesia, Bevan (1935) notes that in another of the survival foci human cases were found during a survey conducted in 1913, and again in yet another focus in 1932; some of the cases then found were acute, others subacute or latent. It has been known for some time that Europeans and natives visiting Gowe had contracted trypanosomiasis; even natives from the surrounding *morsitans* areas, where no indigenous cases occurred, were from time to time infected in this manner.

Bevan found that in laboratory animals of the first passages the Kahondera strain produced a fairly chronic disease, but that its virulence rapidly increased on further passage. Thus two mice infected from the patient lived 52 and 59 days, whereas the next two passages in mice killed one animal in 14 days and a second was moribund after 11 days. Two guinea-pigs inoculated from man lived 119 and 102 days, and a rabbit 21 days. Before its arrival at Entebbe the strain had undergone a long series of passages through laboratory animals.

One of the two mice which brought the strain to Entebbe lived 80 days; the other was killed for subinoculation purposes. On arrival the strain was inoculated into monkeys and white rats, and the following data were recorded (Table I).

Table I

Animal	Total	Duration of disease in days		
		Average	Maximum	Minimum
Monkeys	5	38·3	47	31
White rats:				
Untreated	9	37·4	66	10
Each received 1 dose* of atoxyl	8	30	39	21
White mouse	1	80	—	—

* See Table II.

Three inoculations were made into man. A volunteer (1) received subcutaneously on 2. viii. 35, 1 c.c. citrated heart blood of a mouse infected with the Kahondera strain, and at the same time a control rat was inoculated. The rat showed trypanosomes in its blood for the first time on 6. viii. 35; the volunteer apparently escaped infection. Another volunteer (2) on 9. viii. 35 was inoculated with 1 c.c. of the citrated blood of a monkey infected with the Kahondera strain, a control rat being inoculated at the same time; the rat showed trypanosomes first on 15. viii. 35, the volunteer on 23. viii. 35. Volunteer (1), whose blood 15 days after his first exposure had failed to infect a clean monkey, was on 29. viii. 35 again inoculated, together with a control rat, this time with the heart blood of a rat carrying the Kahondera strain. The rat showed trypanosomes first on 2. ix. 35, the volunteer on 6. ix. 35.

Bevan found the strain in laboratory animals to be susceptible to "Bayer 205", and both volunteers responded readily to treatment with this drug. A full course of the drug was administered, and at the time of writing both men are in excellent health.

These animal reactions show that when first isolated from man the trypanosome was only moderately virulent in laboratory animals, but that on passage its virulence increased. The behaviour of the strain in monkeys on its arrival at Entebbe resembled that of *T. rhodesiense* or the Damba trypanosome rather than *T. gambiense*. In rats the course of disease also suggested *T. rhodesiense*, and in the blood of these animals and in mice enormous numbers of trypanosomes were often present. The adaptation to man, as manifest by the mild course of the disease in Kahondera himself, was considerable, and suggestive of *T. gambiense*, although of course the actual date of the original infection was not ascertainable.

The susceptibility of the strain to arsenic was tested against atoxyl, as shown in Table II. All the nine rats were inoculated on 28. xi. 35 with citrated blood from a monkey infected with Kahondera strain. On 6. xii. 35, when all the animals showed trypanosomes in fresh films, they were treated with atoxyl. The results on the ensuing days are shown in Table II. Where a

fraction occurs, the numerator is the number of trypanosomes seen, the denominator the number of fields examined; where a whole number occurs, it is the number of trypanosomes per fresh field. The left-hand figure in each bracket is the number of trypanosomes seen in the fresh film; the right-hand figure in a thick stained film.

Table II shows that the strain is very much more resistant to arsenic than typical *T. gambiense*, and it is highly probable that this trypanosome in man would not be susceptible to treatment with arsenicals. This test therefore indicates that Kahondera strain is either a *T. gambiense* extremely resistant to arsenic, or *T. rhodesiense*.

Table II

Rat	Weight (in g.)	Dose atoxyl (g. per kg.)	December 6	7	8	9	10	11	12
1	280	0·03	11 —	— —	— —	— —	$\frac{1}{2}$ —	7 —	7 —
2	139	0·03	1 —	$\frac{1}{10}$ —	$\frac{1}{24}$ —	$\frac{6}{10}$ —	12 —	9 —	10 —
3	182	0·05	10 —	$\frac{1}{6}$ —	$\frac{1}{24}$ —	$\frac{1}{24}$ —	$\frac{1}{4}$ —	$\frac{1}{2}$ —	12 —
4	281	0·05	$\frac{1}{2}$ —	— —	— —	— —	— —	— $\frac{3}{100}$	6 —
5	300	0·06	3 —	— —	— —	— —	— —	— —	$\frac{1}{4}$ —
6	114	0·06	1 —	— —	$\frac{3}{8}$ —	$\frac{1}{24}$ —	$\frac{1}{24}$ —	$\frac{1}{4}$ —	6 —
7	220	0·07	3 —	— —	— —	— —	— $\frac{1}{100}$	$\frac{1}{4}$ —	4 —
8	223	0·07	10 —	— —	— —	— —	— —	$\frac{1}{24}$ —	4 —
9 (control)	236	—	3 —	$\frac{1}{10}$ —	$\frac{1}{12}$ —	$\frac{3}{8}$ —	2 —	6 —	10 —

DISPLACEMENT OF THE NUCLEUS

A noteworthy feature of the strain appears to me to be the comparative rarity of pronounced displacement of the nucleus in this trypanosome in the rats and the one mouse examined at Entebbe. Nuclear displacement in a trypanosome is as a rule most readily demonstrable in rats. Slides from nine of these animals have been examined at Entebbe at all stages of their infection, and although trypanosomes were often very numerous in the peripheral blood, exceedingly few typical posterior-nuclear forms have been detected. Among the short forms, which were often present in large numbers, a few showed a tendency towards displacement of the nucleus; but this was very slight, the nucleus lying at the juncture of the posterior and middle thirds of the body of the trypanosome. The highest percentage of typical posterior nuclear forms, i.e. with the nucleus in the posterior quarter of the body, was 4·6 per cent of all trypanosomes seen, and this on a single occasion only.

Bevan refers to anterior displacement as a noticeable feature of the trypanosome in some of the animals he examined. He very kindly sent me for examination blood slides from some of the mice of the early passages from man. In these slides, among the shorter forms of the trypanosome, the tendency to anterior is certainly as common as posterior nuclear displacement, but in the slides I examined the degree of displacement was very slight. In several of the slides volutin granules were very noticeable in the trypanosomes.

The rarity of forms showing typical posterior displacement is indeed a striking feature of this strain; but of course a very much more intensive examination is necessary before a final pronouncement can be made on this matter. So far as the present study is concerned, the rarity of this phenomenon tends to distinguish the Kahondera strain from typical *T. rhodesiense* or *T. brucei*, and is a point of resemblance to *T. gambiense*. The enormous number of trypanosomes present at times in the blood of rats and mice infected with the Kahondera strain is, however, a feature very unusual in *T. gambiense* in these animals, though common enough in guinea-pigs.

Discussion

What then, as Bevan asks, is this trypanosome: is it *T. gambiense* or *T. rhodesiense*? It possesses some of the characters of each, but the weight of the evidence seems to me to point rather towards *T. rhodesiense*. The long association, extending over many years, of man and *morsitans* in the Gowe area; the degree of adaptation of the trypanosome to man; the tendency to reveal the virulence of *T. rhodesiense* when introduced into laboratory animals; the high degree of resistance against arsenic and the susceptibility to "Bayer 205"—all favour *T. rhodesiense*. On the other hand, if this trypanosome is or has recently originated from *T. gambiense* it may fairly be asked why it is so virulent in laboratory animals; and to this question there are two retorts, neither of which at the moment can be gainsaid. The greater virulence might be due (i) to the influence on *T. gambiense* either of residence in game or some other non-human host; or (ii) to the species of tsetse—*G. morsitans* as distinct from *G. palpalis* and *G. tachinoides*, the last two species being the principal transmitters of *T. gambiense* in nature. Both these alternatives are comparatively easy to investigate. There is up to the present no published evidence to show that *T. gambiense* after long residence in ruminants turns into *T. rhodesiense*, or that the repeated transmission of *T. gambiense* by *G. morsitans* produces this result. In fact, recent research into the effect of cyclical transmission on the qualities of a trypanosome tells rather against the likelihood of any such change occurring.

To consider now the other view—derivation from *T. brucei*. The idea of the adaptation of a trypanosome to a particular and in many ways peculiar host—man—presents no inherent improbability. Adaptation of this kind has been observed in *T. vivax* and *T. congolense* in the laboratory, without the main characters of the strain undergoing any change, and there is no sound reason to deny to *T. brucei* the same adaptability when natural conditions favour close contact with man. The fundamental view that *T. gambiense* is a derivative of *T. brucei* seems generally accepted, even by those who believe that *T. rhodesiense* is derived from *T. gambiense*.

In Nigeria a number of varieties of human trypanosomes has been obtained, illustrating all sorts of gradations between the two extreme types. Lester (1933) explains this by supposing these varieties to be stages in the

metamorphosis of *T. gambiense* into *T. rhodesiense*. But in these Nigerian sleeping sickness areas there are three species of tsetse, *G. palpalis*, *G. tachinoides* and *G. morsitans*, two of which, at least, feed freely on game and all of which bite man. In such circumstances there must be excellent opportunity for the exposure of man to the chance of infection with *T. brucei*.

But all this is speculation, and we must fall back on the hope that sooner or later someone will find an opportunity to determine what actually does happen when typical *T. gambiense* lives in a game environment for a year or two and is propagated by a game-tsetse alone. My own investigation of this matter was handicapped first of all by a lack of *G. morsitans* at a time when suitable strains of *T. gambiense* were available, and then, when a supply of this tsetse was for some months forthcoming, by the lack of suitable transmissible strains.

The opening up of Africa that has been a signal feature of the last two or three decades has led to an enormous increase of traffic of all kinds, and natives nowadays travel far and wide. Each year it becomes easier for the supporters of the *gambiense* theory of the origin of *T. rhodesiense* to point to the plausibility of their case. But it must be remembered that the same revolutionary changes in native life will help the spread of *T. rhodesiense*, in man, from hitherto unsuspected sources like Gowe, where biological history is perhaps repeating itself on a miniature scale and *T. brucei* is being constrained to use man.

Moreover, the power of *T. gambiense* to infect man seems to be remarkably stable. Strains "Br" from Prof. J. G. Thomson's laboratory and "Braun" from Hamburg were maintained for 4 and 15 years respectively in the guinea-pig (an animal which has been shown to exert a definite inhibitory effect on the infectivity of *T. rhodesiense* to man) without apparent impairment of this character (Duke, 1935).

The Kahondera strain possesses peculiar interest in this discussion. It seems probable, from Bevan's analysis of the early days of recognized human trypanosomiasis in Rhodesia (1935), that the disease there has been kept alive in a few small isolated foci where game-tsetse persist even in the most unfavourable seasons. Where such a focus coincides with permanent human settlement, the chronic type of *T. rhodesiense* like that described by Kleine at Ikoma should be found—chronic, that is, in terms of man, and adapted to using him as a host.

The argument admits as yet of no finality. The two theories about the origin of *T. rhodesiense* may be mainly of academic interest, but fortunately for those interested in the unravelling of such matters there is also a practical side.

To the dualist who holds that *T. rhodesiense* and *T. gambiense* are one species and different from *T. brucei*, the mere exclusion of infected immigrants coming from *gambiense* territory will keep any inhabited game-tsetse area free from human trypanosomiasis. There will be no autochthonous case; the day

for such is long past. But to the unicisist who believes that *T. brucei* can still do what *T. brucei* has done in the past, there is not only the danger of introducing "ready-made" human strains in man from outside areas, but also danger in allowing man to assume close contact with the game-tsetse and *T. brucei*.

That some strains of *T. rhodesiense* are descended from *T. gambiense* is possibly true, especially in inhabited regions where game-tsetse and the species that feed regularly on man both occur. An arsenic-resistant *T. gambiense* of more than usual activity would be very difficult to distinguish from *T. rhodesiense*; but then the identity of such a strain would itself be open to question.

But in its typical form *T. rhodesiense* is indistinguishable from *T. brucei*, save only in respect of pathogenicity to man; and in the present state of our knowledge it would be mere dogma to assert that *T. brucei* is never able to utilize man as a host. Is there any reasonable doubt that *T. gambiense* was derived, ages ago no doubt, from *T. brucei*, and is at the present day the representative of that widespread species in areas where the tsetse rely largely on man for their supply of mammalian blood? And is it unreasonable to believe that the phenomenon of the adaptation of *T. brucei* to man may still be going on before our eyes wherever man competes with the game as a food supply of *Glossina*?

In attempting to solve this riddle experimentally it is for many reasons easier to approach it through *T. gambiense* than through *T. rhodesiense*. To make, experimentally, *T. brucei* adapt itself to man will necessitate a liberal supply of volunteers; whereas to determine whether *T. gambiense* can become *T. brucei* all that is needed in the earlier stages of the attempt is a supply of game animals and game-tsetse.

The metamorphosis may not, it is true, be reversible, but in all probability it is. Recent experience with *T. rhodesiense* encourages the belief that pathogenicity to man is in certain circumstances not a very stable character; and if *T. gambiense* can adapt itself to game animals, it also may in time lose its normal power of infecting man.

General reflections

The evidence presented in this series, and other previous papers, in my view favours the conclusion that *T. rhodesiense* is more closely related to *T. brucei* than to *T. gambiense*. The opposing view, that it is derived from *T. gambiense*, appears to be based mainly on the power of both these trypanosomes to infect man, and on the absence of definite proof that *T. brucei* can utilize this particular host. But *T. gambiense*, in contrast to *T. rhodesiense*, on its first isolation from man is normally characterized by a low degree of virulence for laboratory animals.

If, therefore, a strain like Kahondera, found in what may be styled a *brucei* environment, is really a descendant of *T. gambiense*, why, in addition to retaining its adaptability to man, should it manifest in other respects the qualities of *T. brucei*?

Variation by *T. brucei* seems to be an equally good explanation of the fact that gradations between *T. gambiense* and *T. rhodesiense* occur in the Nigerian infected areas as variation by *T. gambiense.*

The concluding studies at this Institute strengthen the opinion that *T. rhodesiense* is *T. gambiense* in the making, and that it arises from *T. brucei* when circumstances conduce to broad contact between man and the species of tsetse that feed on game. This belief is compatible with the conviction that as a reservoir of man's trypanosomes the African big game is a factor which with ordinary care can be controlled, though it must not be ignored. It follows from this view that human settlement in game country should be under careful supervision, to insure protection of the population from broad contact with tsetse. Game areas where there are no tsetse are safe, provided they are sufficiently isolated from neighbouring tsetse areas to be secure from invasion by fly. Settlement in proximity to tsetse, if imperative, must be preceded and attended by measures for eradicating the fly in an adequate zone around the settlement, and the food, water and wood supplies must be similarly dealt with. Periodic inspections will be necessary during the early stages of such a settlement, and "Bayer 205" will be useful as a prophylactic until the community is properly established and the breach of contact with the fly complete. To dissociate game from man is merely to anticipate Nature's own decree, for game and flourishing human settlement are incompatible one with the other.

T. gambiense is the principal trypanosome menacing man in Africa, and, compared with its activities in the Congo and Nigeria, the harm done by *T. rhodesiense* is small.

We have seen that the type of *T. gambiense* common in East and Central Africa to-day is not well adapted to life in antelope—very much less so than *T. rhodesiense.* And the little knowledge there is of *T. gambiense* in antelope suggests that this trypanosome might possibly revert gradually to the *brucei* type in this class of mammalian host.

Administrative experience with the Azande tribe in the southern Sudan, and elsewhere, has shown how much can be done by a judicious redistribution of native populations whose normal mode of life brings them into wide contact with tsetse. Once a scattered population has been concentrated in selected areas, the game can be left in occupation of the huge stretches of territory now rendered desolate by denudation, and given over to bush and often tsetse, until such time as portions of that territory are needed for human settlement.

There are and will be for many years to come large areas where game abounds, for the reclamation of which no urgent economic incentive exists.

The actual importance of game to *T. gambiense* in East and Central Africa at the present day is, in all probability, very slight, this trypanosome being primarily if not exclusively a parasite of man, and dependent on man for its survival in nature.

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(*MS. received for publication* 29. III. 1936.—Ed.)

MORPHOLOGICAL AND TAXONOMIC STUDIES ON MAMMALIAN TRYPANOSOMES

III. SPONTANEOUS OCCURRENCE OF STRAINS OF *TRYPANOSOMA EVANSI* DEVOID OF THE KINETONUCLEUS.

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(With 1 Map and 7 Text-figures)

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I. Introduction

As is well known, the kinetoplast, comprising the kinetonucleus and blepharoplast (=basal granule of the flagellum), is one of the most characteristic structures in flagellates of the family Trypanosomidae. There is still no uniformity in the terminology of the two constituents of this apparatus, the names *centrosome* and *blepharoplast* being used both for the basal granule and for the kinetonucleus—usually according to the nationality of the author—while the latter element is sometimes also described as the *parabasal*. The name *kinetonucleus*, which denotes the structure with which we are primarily concerned in this study, is the only one which refers to this organ exclusively, the other three names being applied to cell constituents which have nothing in common with it.

The independent and peculiar nature of the kinetonucleus was recently demonstrated by A. & M. Lwoff (1931) who showed that the trypanosomid flagellates possessed a true parabasal body in addition to the kinetonucleus. All that is known about the kinetonucleus at present is that it is an independent organ of unknown derivation, reproducing itself by binary fission during division of the flagellates. Though typically a permanent structure, in the case of trypanosomes it is possible to produce strains deprived of the kinetonucleus by artificial methods and, moreover, there is evidence that this organ may be lost spontaneously in a previously normal trypanosome strain. It may also be recalled that there exists a "species" (*Trypanosoma equinum*) which is characterized by the total absence of the kinetonucleus, while in other

species of trypanosomes examined from this point of view there is always a certain proportion of individuals lacking this organ. In trypanosomes devoid of kinetonuclei—both in artificially produced and in natural strains—all the vital functions remain unimpaired and such strains behave in every respect like normal ones. It is therefore evident that the kinetonucleus is not indispensable to the existence of these flagellates, though nothing is known precisely regarding its physiological role.[1]

In addition to the purely cytological and physiological interest attached to the kinetonucleus, it has acquired some importance in chemotherapy, for it was shown that certain drugs have a specific action upon this organ. Attention has also been directed to the kinetonucleus from the genetic point of view, since its absence in a certain number of individuals of normal strains, its spontaneous disappearance in such strains, and the existence of an established race of trypanosomes naturally devoid of this organ, all represent parts of a single problem, concerned with the mechanism of this phenomenon and with the origin of races thus modified.

In the present paper an account is given of the spontaneous occurrence of strains devoid of a kinetonucleus in *T. evansi* from the Anglo-Egyptian Sudan. While one of us (S. C. J. B.) conducted all the field observations on the spot, the other (C. A. H.) is responsible for the subsequent treatment of material from this locality, and also from other sources.

Though we do not profess to have solved the question regarding the significance and origin of races without kinetonuclei, this first record of the occurrence of such aberrant strains under natural conditions advances our knowledge of the question a step further and provides data which might serve to stimulate further investigations in this direction on the part of other workers.

Before proceeding with our own observations we propose to review briefly the facts already known regarding the loss of the kinetonucleus in trypanosomes.

II. Critical review of previous work

The starting point of all investigations on the loss of the kinetonucleus in trypanosomes goes back to 1910, when Werbitzki demonstrated that certain organic dyes (having Ehrlich's orthoquinoid constitution) when allowed to act upon trypanosomes cause the disappearance of the kinetonucleus, without affecting their vitality. These results were later confirmed and extended by a number of other workers.[2]

The direct action of the dyestuffs upon the kinetonucleus was demonstrated by the fact that in trypanosomes treated both *in vitro* and *in vivo* this organ

[1] For further particulars regarding the cytology of the Trypanosomidae the reader is referred to the memoir by A. & M. Lwoff (1931).

[2] We do not propose to enter into all the details of the earlier investigations, exhaustive critical reviews of which were published by Dobell (1912) and by Roudsky (1923: this posthumous publication covers the work done up to 1914), to whose memoirs the reader is referred for fuller details and for the bibliography.

alone becomes stained, and by the observation that normal mice and those infected with a strain of trypanosomes deprived of the kinetonucleus are more susceptible to the toxic action of the dyestuffs than are mice infected with trypanosomes in which this organ is intact. This difference was attributed to the partial absorption by the kinetonuclei of the dyestuff present in the blood (Roudsky, 1923).

The proportion of trypanosomes that can be deprived of the kinetonucleus through the action of dyes varies according to species: whereas repeated treatment of *T. brucei* and *T. evansi* may result in a universal disappearance of this organ, in *T. dimorphon* and *T. congolense* the maximum percentage in which removal of the kinetonucleus could be obtained was from 32 to 50, while in *T. lewisi* only 17 per cent lost this organ.

As regards the persistence of the "akinetonucleate"[1] condition, there seems to be no unanimity on this question. It was established that if the removal of the kinetonucleus does not involve all the individuals, the percentage of akinetonucleate trypanosomes gradually decreases and the strain soon becomes normal again. However, when the disappearance of the kinetonucleus is universal among the trypanosomes, the strain may persist in the akinetonucleate condition for years.[2] Some workers believed that this condition could become permanently established, but Laveran (1919) described a case in which a strain of *T. evansi* remained without the kinetonucleus for seven years, after which this organ reappeared and the strain became normal. However, A. & M. Lwoff (1931) have criticized the method of observation on which this case was based, and it would seem that the question of the permanence of artificially produced akinetonucleate strains remains open.

Opinions are also divided regarding the mechanism causing the kinetonucleus to disappear. Some believed that this organ was not removed at all, but was rendered unstainable by the action of the drugs; others attributed its disappearance to direct destruction by the chemicals; while others again thought that the production of the akinetonucleate state is due to irregular division of the trypanosomes, in the course of which the kinetonucleus fails to divide, with the result that this structure is retained by one of the daughter individuals, while the other one lacks it; it was also suggested (by Kudicke, 1911) that the production of an akinetonucleate strain may be due to selection by the drug of the akinetonucleate individuals, usually present in normal strains, which might be more resistant to its action.

Most of the evidence available at present appears to be in favour of two methods of elimination of the kinetonucleus: by its direct destruction by the drug, all the stages of which have been observed (cf. Roudsky, 1923), and by

[1] Trypanosomes deprived of the kinetonucleus have been called "ablepharoplastic", "acentrosomic" or "aparabasal", which are all inappropriate: "akinetonucleate", though a clumsy term, is at least unambiguous.

[2] However, as will be shown later (p. 55), it is doubtful if the kinetonucleus is ever absent in *all* the trypanosomes, at least in the case of naturally occurring akinetonucleate strains.

irregular division. Robertson (1929) has recently shown that both these methods may be responsible for the production of akinetonucleate individuals in *Bodo*. It is conceivable that they may be effective singly or in combination, the drug either destroying the kinetonucleus before the trypanosome divides, or depriving this organ, entirely or partially, of the power to divide. In the latter case the result will be the production of akinetonucleate individuals in progressively increasing numbers. If it is assumed that the aberrant trypanosomes arise from normal ones owing to irregular division of the kinetonucleus in the latter, then in the progeny of each normal trypanosome thus affected the ratio of akinetonucleate individuals to normal ones will be $(2^n - 1) : 1$, where n = number of generations. It can easily be seen that if a large number or all individuals are affected in this way by the drug, irregular division alone would be capable of rapidly producing an akinetonucleate strain. This has been convincingly demonstrated by Jírovec (1929), in the case of *T. evansi*, and—as will be shown later—the same phenomenon accounts for the presence of the individuals devoid of kinetonuclei in normal strains of trypanosomes.

We have hitherto considered akinetonucleate strains of trypanosomes produced artificially by the action of chemicals. We now turn to the first record of the spontaneous transformation of a normal strain of trypanosomes into an akinetonucleate strain. This case, described by Wenyon (1928), refers to a strain of *T. evansi* (= *T. marocanum*) kept in laboratory animals since 1922. Some time between 1926 and 1927 the strain which had previously exhibited the normal morphology of *T. evansi* became totally akinetonucleate and indistinguishable from *T. equinum*. This strain is still being maintained at the Wellcome Bureau of Scientific Research, the akinetonucleate condition (Fig. 5) having persisted already for about nine years (cf. Table I, Nos. 8–12). As stated by Wenyon (1928) the precise time when the change took place is not known; it is clear, however, that it was spontaneous and not due to any action of drugs.

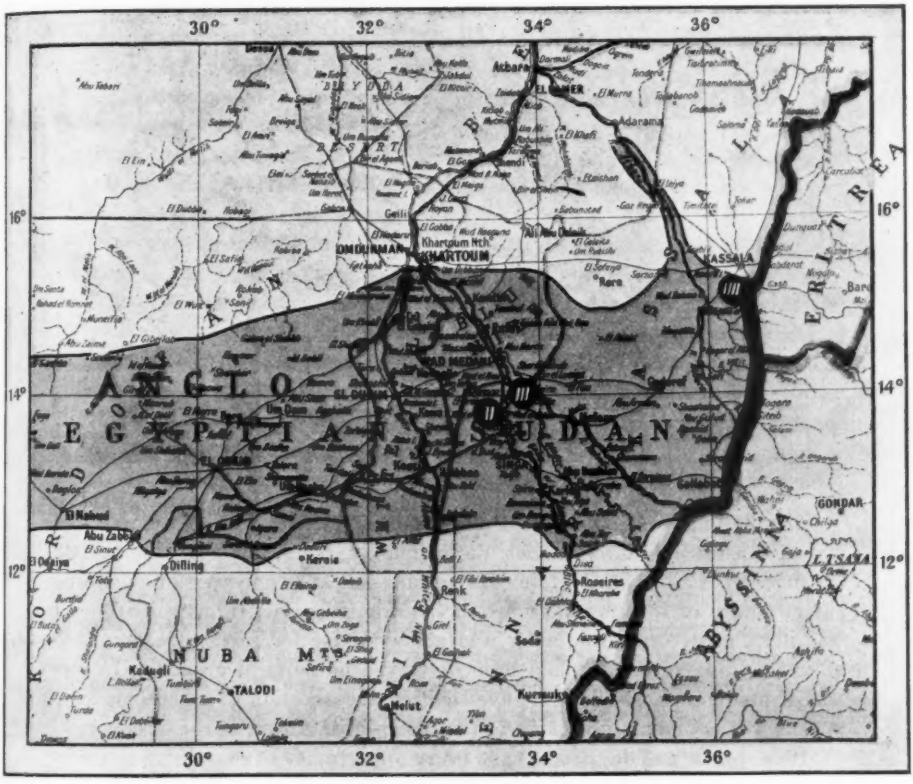
This concludes the record of previous observations on akinetonucleate strains of trypanosomes, some aspects of which will be further discussed in the succeeding sections.

III. Akinetonucleate strains of the camel trypanosome

The akinetonucleate strains of trypanosomes recorded up to the present were either experimentally produced or occurred in unnatural hosts under laboratory conditions. Though there is no doubt that the strain of *T. evansi* described by Wenyon (1928) made its appearance spontaneously and had not been previously subjected to treatment with drugs, there was always the possibility that the artificial conditions under which the strain was maintained might have been themselves instrumental in modifying the structure of the trypanosomes. However, the observations recorded below, which were carried out on the trypanosome of Sudanese camels, provide satisfactory evidence

that akinetonucleate strains can arise spontaneously under perfectly natural conditions.

The distribution of *T. evansi* (sometimes described as *T. soudanense*) in the Anglo-Egyptian Sudan is shown in the accompanying map by the shaded portion, representing the "belt" in which infected camels are regularly to be found in any rainy season (usually from the middle of June to the end of September). This enzootic area is subject to a certain amount of variation in different years and may extend for about one degree of latitude further north

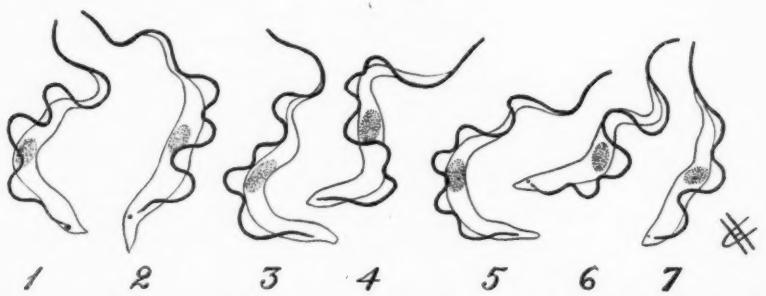


Distribution of camel trypanosomiasis in the Anglo-Egyptian Sudan. (The shaded portion denotes the enzootic area of *T. evansi*; the numbered black circles indicate the localities in which the akinetonucleate strains—Cases II, III and IIII respectively—have occurred.)

and south. Furthermore, odd cases of trypanosomiasis also occur well outside these limits, but the majority of them are attributable to the wide range over which the camels travel from the foci of infection.

The camel trypanosome of the Sudan has the typical structure of *T. evansi* (Fig. 1) and is indistinguishable from other strains of this species from various

hosts and countries. In January, 1934, a "clean" camel which had been kept in Khartoum (outside the trypanosomiasis "belt") for several years was inoculated with trypanosomes from a naturally infected camel. The trypanosomes in the latter were very scarce and their structure appeared to be normal, though this point was not specially noted at the time. However, on examination of stained blood-films from the passage camel (here referred to as Case I), it was found that practically all the trypanosomes were devoid of the kinetonucleus (Figs. 3, 4) and were indistinguishable from the akinetonucleate laboratory strain of *T. evansi* described ~~below~~ above (Fig. 5) or from *T. equinum* of South American horses.



Figs. 1–7. Kinetonuclear variations in *Trypanosoma evansi*. 1, form with full-size kinetonucleus ("*T. soudanense*": normal cameline strain "R"); 2, form with reduced kinetonucleus ("*T. soudanense*": normal cameline strain "EAC"); 3, 4, forms devoid of kinetonuclei ("*T. soudanense*": akinetonucleate cameline strain "Case I"); 5, form devoid of kinetonucleus ("*T. marocanum*": akinetonucleate laboratory strain); 6, 7, forms with reduced kinetonuclei ("*T. venezuelense*": normal strain).

This finding of an akinetonucleate strain of *T. evansi* in its natural host was of considerable interest, but, unfortunately, it did not help to elucidate the mode of origin of such strains, for nothing was known for certain regarding the structure of the trypanosomes in the original camel from which Case I was inoculated, while the latter was not available for further observations after this discovery, as it had been destroyed on surgical grounds. In this way a unique opportunity for investigating the question of akinetonucleate strains was lost, mainly because of the unexpected nature of the discovery.

However, it was decided to take every opportunity of examining as many cases of camel trypanosomiasis as possible, with the object of detecting further akinetonucleate strains and of throwing some light on their mode of origin. Though thousands of cases had previously been examined by one of us (S. C. J. B.) nothing unusual was ever detected in their structure, but it is conceivable that akinetonucleate strains, if present, may have been overlooked in the course of routine examinations conducted mainly for therapeutic or immunological purposes. However, following the finding of the akinetonucleate strain particular attention was devoted to the morphology of the trypanosomes seen. During the last two years the examination of more than

one hundred cases of camel trypanosomiasis resulted in the discovery among them of *three* more akinetonucleate strains. Though blood films from these cases were obtained, it was again impossible to continue the investigations on these camels, since they had been treated locally as soon as the diagnosis of the infection was established.

The particulars of these cases are as follows:

Case II: naturally infected camel from Wad-el-Haddad (see Map).

Case III: naturally infected camel from Shabarga (see Map). Both Cases II and III were camels belonging to native owners, and nothing is known regarding their previous history. They have since been treated and lost sight of.

Case IIII: naturally infected camel from the vicinity of Kassala (see Map). This camel belonged to the tribesmen; it was born and reared locally and had never been previously treated.

It is thus seen that the last three cases of akinetonucleate strains of *T. evansi* occurred spontaneously in *natural infections* of camels, whereas Case I was from a passage animal.

The exact proportion of akinetonucleate trypanosomes was estimated for Cases I, II and III by a careful examination of 500 individuals in each strain. It was found that in each case a very small number of individuals still retained kinetonuclei of variable dimensions (as in Figs. 2, 6, 7), the proportion of individuals lacking this organ entirely being from 97·0 to 99·4 per cent (see Table I). The significance of this fact will be discussed in Section V.

As regards the geographical distribution of Cases II, III and IIII, a glance at the accompanying map shows that they were not localized in any particular focus, but occurred in widely separated localities within the enzootic area of camel trypanosomiasis.

The three cases of natural infections with akinetonucleate strains represent less than 3 per cent of the total number of infected camels examined since 1934, the trypanosomes in all the other cases having the typical structure. A number of these camels were kept under continuous observation for long periods of time, without any deviation from the normal type taking place among the trypanosomes. On several occasions strains of typical trypanosomes were inoculated from camel to camel, from camel to gerbil, and from gerbil to gerbil, but in every instance the trypanosomes remained unaltered. The last two sets of observations were carried out (1) in the hope that a prolonged study of the infected camels might result in the detection of an akinetonucleate strain of the trypanosome *in statu nascendi*, and (2) in order to ascertain whether this change in the trypanosomes could be brought about by passages from mammal to mammal. As was stated above, the results were negative, the trypanosomes remaining true to type. However, to have obtained a positive result would, in view of the probabilities, have been an exceptional piece of luck.

The fact that the kinetonucleus can be eliminated from the trypanosomes, and a strain devoid of this organ produced by the action of chemicals, led us

to consider the possible effect of therapeutic treatment upon the occurrence of akinetonucleate strains of the camel trypanosome, though there was no reason to believe that "Naganol" (="Bayer 205"), which is used for the treatment of camel trypanosomiasis in the Anglo-Egyptian Sudan, had any such effect. In fact, Leupold (1925) has demonstrated that this drug does not attack the kinetonucleus at all. In strains resistant to "Bayer 205" trypaflavine caused the disappearance of the kinetonucleus of trypanosomes in the same way as in normal strains, whereas in arsenic-resistant strains similar treatment left this organ unaltered. Leupold concluded that, like the dyestuff, arsenicals become attached to the kinetonucleus, which is then no longer susceptible to the action of trypaflavine, whereas "Bayer 205" has no affinity for this organ, leaving it free to be acted upon by this dyestuff.

Nevertheless, since only one of our akinetonucleate strains (Case IIII) was entirely above suspicion as regards previous treatment, this factor had to be taken into consideration. The data available on this subject are as follows. A careful examination of over thirty infected camels, which had undergone treatment some time during the preceding few years, revealed only normal trypanosomes in the blood. More direct evidence was provided by numerous cases which relapsed following treatment with insufficient doses of the drug, and also by a number of cases which had been definitely cured and subsequently experimentally reinfected (in the course of immunological studies). In all these cases the trypanosomes were unaltered. There is thus satisfactory evidence that treatment with "Naganol" does not cause the disappearance of the kinetonucleus in the trypanosomes.

It is thus seen that, apart from the negative evidence regarding the effect of drug treatment, nothing is known regarding the conditions under which the akinetonucleate strains of trypanosomes originate in nature. However, the very fact that such strains do arise spontaneously is significant and may help to throw light on the origin of permanently established akinetonucleate races or species, such as *T. equinum*. Further data bearing on the same problem are provided by the presence of individuals devoid of kinetonuclei in otherwise normal strains of trypanosomes, which are dealt with in the next section.

IV. Akinetonucleate forms in normal strains

The artificial production of akinetonucleate strains of trypanosomes drew attention to the fact that a certain proportion of trypanosomes devoid of the kinetonucleus occur in normal strains of these flagellates. This was first noted by Kudicke (1911) who found that 5 per cent of the trypanosomes (*T. brucei*) examined by him lacked kinetonuclei, whence he concluded that these trypanosomes were selected by the drugs and gave rise to akinetonucleate strains. However, this was a mere conjecture.

More recent contributions to this question were made by Lavier (1927, 1928, 1928 *a*, 1929), Jírovec (1931), and by Nattan-Larrier & Noyer (1931,

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Parasitology, Vol. 29, No. 1, 1937, p. 43.

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Page 48: Line 9, *for* below *read* above.

Page 51 (Table I), (Serial No. 2), Last (8th) Column, Line 3, *for* ,, *read* C.A.H.

,, (Table I), (Serial No. 27), Last (8th) Column, Line 29, *for* ,, *read* C.A.H.

,, (Table I), (Serial No. 36), Last (8th) Column, Line 40, *for* ,, ,, *read* Lavier (1927).

,, (Table I), (Serial No. 37*a*), Last (8th) Column, Line 43, *for* ,, ,, *read* Jírovec (1931).

,, (Table I), (Serial No. 42), Last (8th) Column, Line 49, *for* ,, *read* C.A.H.

Table I. *Proportion of akinetonucleate forms in the pathogenic trypanosomes*

Table I. ***Proportion of akinetonucleate forms in the pathogenic trypanosomes***

Serial No.	Species	Variety and/or strain	Locality	Source of trypanosomes*	Percentage of forms without kinetonucleus	No. of trypanosomes examined	Observers
1	*T. evansi*	—	Java	Equine (gp.: 1st p.)	2·3	2000	C.A.H.
1*a*	,,	—	,,	,, (m.)	1·0–8·0	?	Krijgsman (1930)
2	,,	—	India (Muktesar)	,, (nat.)	0·3	1000	,,
3	,,	"*su-auru*"	Middle Asia (Uralsk)	Cameline (nat.)	5·0	500	,,
4	,,	,,	,, (Ashkhabad)	,, (nat.: 2 cases)	0·6 & 0·8	500 + 500	,,
5	,,	*aegyptum*	Egypt	,, (gp.)	0·0	—	Nattan-Larrier & Noyer (1932)
6	,,	*marocanum* var. *cameli*	Morocco (Marrakech)	,, (gp.)	2·7	—	,, (1931)
7	,,	,,	,, ,,	,, (nat.)	8·0	500	Lavier (1928 *a*)
8	,,	,, (Velu)	,, —	Equine (gp.)	8·0	500	,, ,,
9	,,	,, ,,	,, —	,, (gp.: 1926)	0·6	500	C.A.H.
10	,,	,, ,,	,, —	,, (gp.: 1927)	**99·4**	500	,,
11	,,	,, ,,	,, —	,, (m.: 1933)	**99·2**	500	,,
12	,,	,, ,,	,, —	,, (m. & gp.: 1936)	**99·8**	500 + 500	,,
13	,,	,, (Sfax)	,, —	,, (lab.)	0·4	1000	Lavier (1928 *a*)
14	,,	,, —	,, (Missour)	,, (lab.: recent)	0·9	—	Nattan-Larrier & Noyer (1931)
15	,,	,, —	,, ,,	,, (m.: 25th p.)	0·3	—	,, ,,
16	,,	,, —	,, ,,	,, (m.: 87th p.)	4·3	—	,, ,,
17	,,	*soudanense* (R)	Sudan (Tokar)	Cameline (nat.: 3 cases)	1·0	600 + 600 + 900	C.A.H.
18	,,	,, (EAC)	,, (Dinder)	,, (nat.)	4·4	500	,,
19	,,	,, (A)	,, —	,, (nat.)	5·4	500	,,
20	,,	,, (B)	,, —	,, (nat.)	6·0	500	,,
21	,,	,, (I)	,, (Khartoum)	,, (exp.)	**97·0**	1000	,,
22	,,	,, (II)	,, (Wad-el-Haddad)	,, (nat.)	**99·4**	500	,,
23	,,	,, (III)	,, (Shabarga)	,, (nat.)	**99·0**	500	,,
24	,,	—	British Somaliland	,, (nat.)	0·4	500	,,
25	,,	—	Mauritius	? (lab.)	0·4	1000	Lavier (1928 *a*)
26	,,	—	,,	Bovine (nat.: 2 cases)	0·3 & 0·5	1000 + 1000	C.A.H.
26*a*	,,	—	?	? (gp.)	1·6–2·2	?	Jirovec (1931)
27	,,	*hippicum*	Panama	Equine (gp.)	4·0	500	,,
28	,,	*venezuelense*	Venezuela	,, (lab.)	16·0–26·0	—	Lavier (1929)
29	,,	,,	,,	,, (m.)	30·2	500	C.A.H.
30	,,	*equinum*	South America	,, (m.)	**98·6**	500	,,
31	*T. equiperdum*	—	Algeria	,, (lab.)	2·6	500	Lavier (1928 *a*)
31*a*	,,	—	?	,, (m.)	0·1– 2·5	?	Jirovec (1931)
32	*T. brucei*	—	—	? (m.)	5·0	—	Kudicke (1911)
33	,,	—	Tanganyika (Ikoma)	Bovine (lab.)	5·8– 9·4	—	Lavier (1927)
34	,,	—	Uganda (Mbarara)	Canine (lab.)	6·0–10·0	—	,, ,,
35	,, (?)	—	,, (Damba)	Antelope (lab.)	1·0– 2·0	—	,, ,,
35*a*	,,	—	?	? (m. & gp.)	0·6– 2·2	?	Jirovec (1931)
36	*T. rhodesiense*	—	Tanganyika	Human (lab.)	1·0– 2·0	—	,, ,,
36*a*	,,	—	?	,, (gp. & m.)	0·3– 0·8	?	,, ,,
37	*T. gambiense*	—	Uganda	,, (lab.)	0·5– 2·0	—	,, ,,
37*a*	,,	—	Fernando Po (*et alibi*)	,, (r. & m.)	0·01–6·0	?	,, ,,
38	*T. vivax*	—	,,	Bovine (nat.)	0·1	1000	C.A.H.
39	,,	—	Nigeria	,, (nat.)	0·2	500	,,
40	*T. congolense*	—	Uganda	Feline (exp.)	0·1	1000	,,
41	,,	—	,,	Bovine (nat.)	0·4	500	,,
41*a*	,,	—	?	,, (gp. & m.)	0·1–0·8	?	Jirovec (1931)
42	*T. simiae*	—	Belgian Congo	Porcine (nat.: 2 cases)	0 & 0·8	500 + 500	,,

* The original strain is indicated outside the brackets; the actual source of the trypanosomes examined and other information regarding the strains appear within the brackets, with the following abbreviations: exp. = experimental infection; gp. = guinea-pig strain; lab. = laboratory strain (unspecified); m. = mouse strain; nat. = natural infection; p. = passage; r. = rat strain. The years refer to the date when the preparation was made.

1932), who estimated the proportion of akinetonucleate forms in various strains of *T. evansi*, *T. equiperdum*, *T. brucei*, *T. gambiense* and *T. rhodesiense*.

During the last three years one of us (C. A. H.) has collected abundant material on the morphology of various species and strains of mammalian trypanosomes from different parts of the world.[1]

The opportunity was taken to calculate the proportion of akinetonucleate trypanosomes in these preparations, with the object, on the one hand, of extending the records to species not dealt with by previous workers (*T. vivax* and *T. simiae*), and, on the other hand, of amplifying the data already available, especially with regard to *T. evansi*, of which alone twenty-four additional strains have been examined.

The results of this study, together with previous records, are brought together in the accompanying Table I, which also includes the corresponding figures for the akinetonucleate strains described in this paper and by Wenyon (1928).

This table, representing the most comprehensive survey of the subject hitherto published, reveals a number of interesting facts. If the natural groups of species (cf. Hoare & Coutelen, 1933) are compared, it will be seen that akinetonucleate forms are the least prevalent in members of the *vivax* and *congolense* groups, the percentage of such individuals in *T. congolense* and *T. simiae* varying from 0 to 0·8, and in *T. vivax* from 0·1 to 0·2.[2] In the *brucei* group (*T. brucei*, *T. rhodesiense*, *T. gambiense*) the number of akinetonucleate forms reaches a much higher figure, their proportion varying from 0·01 to 10·0 per cent. However, the greatest range of variation and the highest percentage of individuals lacking this organ is attained in the *evansi* group and especially in *T. evansi*.[3]

In this species the proportion of akinetonucleate forms in strains from different parts of the world and in different hosts varies from 0 to practically 100 per cent (*T. equinum*). However, a comparison of the figures for the New and Old Worlds reveals a marked difference. If we exclude the akinetonucleate strains of the camel trypanosome, as being aberrant and of sporadic occurrence, it will be found that the *normal* proportion of forms lacking kinetonuclei in strains of the Old World varies from 0 to 8·0 per cent, while in the New World the incidence of such forms is considerably higher, varying from 4·0 to 100 per cent (cf. Table I, Nos. 27–30). Moreover, there appears to be a certain

[1] The preparations were kindly provided by the following gentlemen, whose help is very gratefully acknowledged: Dr A. R. D. Adams (Mauritius), Mr A. V. Bogoroditsky (Turkestan), Dr G. C. Bourguignon (Belgian Congo), Prof. C. Bubberman (Java), Mr J. Carmichael (Uganda), Dr G. N. Hall (Nigeria), Dr D. Sassuchin (U.S.S.R.), Dr J. Schwetz (Belgium), Mr W. Taylor (India) and Dr C. M. Wenyon (London).

[2] It is interesting to note that trypanosomes of the *congolense* group also proved to be the least susceptible to the removal of the kinetonucleus when treated by dyestuffs (cf. p. 45).

[3] We regard all the trypanosomes having the morphological characters of *T. evansi* as belonging to this species (cf. p. 54). The names under which some of them have been described are given in the third column of the Table.

gradation in the distribution of the American races of *T. evansi*, the akinetonucleate condition becoming more pronounced as one proceeds from north to south. Thus, "*T. hippicum*", occurring in Central America and in Colombia, is 4·0 per cent akinetonucleate; in Venezuela it is replaced by "*T. venezuelense*", which has from 16·0 to 30·2 per cent akinetonucleate forms, while in the rest of South America the prevalent race "*T. equinum*" is totally akinetonucleate. As regards the Old World races of *T. evansi*, the proportion of individuals devoid of kinetonuclei does not vary appreciably in strains from different countries: in Eastern Asia (Nos. 1, 2) the percentage is from 0·3 to 2·3; in Middle Asia (Nos. 3, 4) from 0·6 to 5·0; and in North and North-East Africa (Nos. 5–24) from 0 to 8·0. It would also appear that this condition is not influenced by the nature of the host, since the percentage of akinetonucleate forms in the equine strains (0·3–8·0) is comparable to that in the cameline ones (0–8·0). However, within the same strain the number of individuals devoid of kinetonuclei may vary considerably at different periods (cf. Table I, Nos. 8, 9 and Nos. 14, 15, 16). As regards the camel trypanosome of the Sudan, the percentage of forms without kinetonuclei from different cases varied from 1·0 to 6·0 (cf. Table I, Nos. 17–20), which is well within the range recorded for other races of *T. evansi*. There is thus no evidence, such as exists in the case of the American races, of any tendency for a local increase in the number of trypanosomes lacking kinetonuclei which might account for the appearance of the totally akinetonucleate strains.

V. GENERAL CONSIDERATIONS

In the foregoing sections an account has been given of the existing data regarding the loss of the kinetonucleus in trypanosomes, produced experimentally or occurring spontaneously, both under artificial and natural conditions.

In the case of the strains produced experimentally it would appear that the kinetonucleus is destroyed directly or incapacitated for further reproduction by the action of chemicals (cf. p. 45), whereas in the case of the spontaneously occurring strains, the agency causing the kinetonucleus to disappear remains unknown.

As regards the mechanism by which this organ is eliminated, there is a considerable amount of evidence that failure of the kinetonucleus to divide is chiefly responsible for this phenomenon (cf. p. 46). Irregular division of the kinetonucleus has been observed under both experimental and natural conditions. It was first described by Werbitzki (*vide* Dobell, 1912) and later by Kudicke (1911) and Jírovec (1929) in artificially produced akinetonucleate strains, while Lavier (1928) demonstrated its occurrence in normal strains of trypanosomes and attributed the prevalence of akinetonucleate individuals in such strains to this phenomenon. In the course of this work we have also had occasion to observe abnormal division of this type, and we are fully in agreement with Lavier's interpretation.

It would seem that the same phenomenon could account for the spontaneous production of totally akinetonucleate strains such as that described by Wenyon (1928) and those dealt with in the present paper, though the cause of the *universal* disappearance of the kinetonucleus in a strain is still a mystery. It would seem, however, that in order to bring about this condition this agency—whatever its nature—must influence all or most of the trypanosomes present simultaneously. On the other hand, it is conceivable, on theoretical grounds, that an akinetonucleate strain might arise from a normal one through the chance inoculation by the natural vector of a single trypanosome devoid of the kinetonucleus.[1] However, this method would apply only in the case of akinetonucleate strains occurring under natural conditions, such as the cameline ones described in this paper; it would not account for the production of the laboratory strain of *T. evansi* ("*marocanum*"), where numerous trypanosomes are inoculated at each passage.

The question now arises as to the duration of the aberrant condition in the akinetonucleate strains. The general tendency is to regard this condition as permanent, and, indeed, the facts available show that akinetonucleate strains produced artificially can persist for years (cf. p. 45), while the spontaneously produced akinetonucleate laboratory strain of *T. evansi* has also remained unaltered for the last nine years. To these may be added *T. equinum*, as an example of an established akinetonucleate race of trypanosomes.

Though the origin of *T. equinum* is unknown, it has long been regarded as being closely allied to *T. evansi*. The ease with which akinetonucleate strains can be produced artificially has led Wenyon (1926) to regard *T. equinum* as a race of *T. evansi*, while Lavier (1929) refers to it as a local variety of this species, with which (="*T. hippicum*")—from the point of view of the proportion of akinetonucleate forms—it is connected by an intermediate variety, "*T. venezuelense*" (cf. Table I, Nos. 27–30).

The spontaneous appearance of akinetonucleate strains of the camel trypanosome, described in the present paper, lends further support to this view. From what is known about other akinetonucleate strains it is not improbable that, under suitable conditions, such "natural" strains might be capable of "breeding true", without kinetonuclei, and that *T. equinum* may have originated in this manner.

The occurrence in various parts of the world of strains of trypanosomes indistinguishable from *T. evansi* (the names of some of which appear in the third column of the table), but differing from each other only in the proportion of individuals devoid of kinetonuclei, which varies from 0 to about 100 per cent, shows that they all belong to a single species, *T. evansi*.[2] In view of the structural peculiarity of "*T. equinum*" and of the fact that there is no

[1] This hypothesis lends itself to experimental verification, by the inoculation of single trypanosomes into laboratory animals.

[2] The principles of the classification of the trypanosomes adopted here have been discussed elsewhere (Hoare, 1936) and need not be repeated.

morphological gap between this race and the typical *T. evansi* ("*T. hippicum*"), since they are connected by an intermediate race, "*T. venezuelense*", "*T. equinum*" may be given subspecific status.

In Section II it has been pointed out that persistence of the akinetonucleate condition was observed in the experimentally produced strains only when the loss of the kinetonucleus involved *all* the trypanosomes, whereas in the presence of a certain proportion of normal individuals the strain gradually regained its normal constitution (cf. p. 45). It is doubtful, however, if any strain ever becomes totally akinetonucleate, but even if it does do so it is impossible to assert that the kinetonucleus cannot arise *de novo* in trypanosomes deprived of this structure. It has already been shown (p. 49 and Table I, Nos. 21–23) that a careful examination of the cameline strains revealed the presence of a kinetonucleus in from 0·6 to 3·0 per cent of individuals. Similar examinations of the "*marocanum*" strain at different periods have shown that this organ was present in from 0·2 to 0·8 per cent (cf. Table I, Nos. 10–12), while in "*T. equinum*" 1·4 per cent of the trypanosomes were found to possess it. Its dimensions varied considerably, ranging from full size to a minute dot (as in Figs. 2, 6, 7). Kinetonuclei of reduced size were also seen in normal strains of *T. evansi*, being especially common in "*T. venezuelense*" (Figs. 6, 7). A dwindling in the size of the kinetonucleus "giving the impression of a veritable atrophy" was previously noted in trypanosomes of the *brucei* group by Lavier (1928).

The significance of these kinetonuclei is not clear. While in normal strains of trypanosomes they may possibly represent atrophied structures, their presence in akinetonucleate strains of long standing requires some other explanation. It is conceivable that the kinetonuclei seen in these cases are the result of some regenerative activity on the part of the trypanosome, but the failure to re-establish the normal structure throughout the strain indicates that the factors responsible for the original disappearance of the kinetonuclei may still be operative.

However, since the factors causing the elimination of this organ, and its derivation in the body of the flagellate are unknown, these and other related questions dealt with in the present paper remain unsolved pending further investigations.

VI. SUMMARY

An account is given of the spontaneous occurrence in the Anglo-Egyptian Sudan of strains of the camel trypanosome, *Trypanosoma evansi*, devoid of kinetonuclei.

These cases, of which four are recorded, were observed in widely separated localities within the enzootic area of camel trypanosomiasis.

A critical review is given of the question of "akinetonucleate" trypanosomes in general—in strains produced experimentally and in those occurring

spontaneously—with special reference to their mode of origin and their significance in the production of new races.

The prevalence of "akinetonucleate" forms in normal strains of trypanosomes has been estimated in the case of a number of species and races hitherto not examined from this point of view, all the existing data being brought together in tabular form.

While the factors causing the appearance of "akinetonucleate" individuals and strains under natural conditions could not be ascertained, irregular division of the trypanosomes is held to be mainly responsible for the elimination of the kinetonucleus and for the persistence of the aberrant condition.

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(*MS. received for publication* 29. IV. 1936—Ed.)

FURTHER OBSERVATIONS UPON *ENTAMOEBA AULASTOMI* NÖLLER

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(With Plates II and III, containing Figs. 1–54)

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I. Introduction

Since 1924, when Boeck and Drbohlav first cultivated *Entamoeba histolytica*, many workers have studied the entamoebae of warm-blooded hosts. As a result of the series of brilliant papers upon the intestinal Protozoa of monkeys and man, published by Dobell during the last decade, the life-history of *E. histolytica* and the cultural conditions under which it can live are known in detail. But little is known of the entamoebae from invertebrate hosts, and it has been my aim to study such a parasite as intimately as possible in order to compare it with the entamoebae of vertebrate hosts and with *E. histolytica* in particular. *E. aulastomi*, found in the gut of the horse-leech (*Haemopis sanguisugae*) and first cultivated by Drbohlav (1925), was chosen for this study.

II. Methods

On 19. vi. 31 a strain of *Entamoeba aulastomi* was isolated from the gut of a horse-leech. It has been growing in cultures containing this species of protozoon only, together with mixed bacteria, ever since. The method of cultivation, division, and cyst formation have been described previously (Bishop, 1932). *E. aulastomi* was grown, at room temperature (18–22°C.), in media consisting either of inactivated horse serum diluted 1 : 10 in 0·5 per cent NaCl solution or of an inspissated horse-serum slope covered with the liquid medium. To each type of medium a little sterile, solid rice starch was added before inoculation. During the first year of subcultivation large crops of cysts were

formed from the 9th to the 14th day after inoculation in both types of medium, but later the rate of cyst production fell off in the wholly liquid medium, and no more cysts appeared in the part-liquid part-solid medium. It was found, however, that if the amoebae were grown for 4–7 days in the part-liquid part-solid medium with plenty of starch and then subcultivated in the wholly liquid medium with only a trace of starch, large crops of cysts appeared between the 14th and 20th days from the time of inoculation. This method has been used during the last 2½ years and still gives excellent results. In many tubes few or no free amoebae could be found, almost every individual having encysted.

Dobell (1927) found that the maximal time of survival at room temperature for the starch-fed amoeboid form of *E. histolytica* was 3 days. When studying the excystation and metacystic development of this amoeba he (Dobell, 1928) was able to obtain cysts free from amoebae by leaving cultures containing cysts and amoebae on his bench for about a week. He also freed culture cysts from concomitant amoebae by treating the mixture with *N*/20 HCl for 30 min. at room temperature, or for 75 min. at 37° C.

But with *E. aulastomi* the attempts to obtain healthy cysts free from amoebae met with many difficulties. The deposits containing large crops of 4-nucleate cysts and few amoebae were sedimented in sterile 0·5 per cent NaCl solution, or sterile distilled or tap water, and kept at either room temperature or at 2°C. for varying periods of time, but in every case the majority of cysts had died before the amoebae totally disappeared. Though in some cultures every amoeba appeared to have encysted this could not be proved, and it was not safe to assume that not a single free form was present. At 37°C. cysts died as rapidly as the amoeboid stage. In my earlier paper it was shown that at room temperature all amoebae were killed in less than 10 min. if treated with *N*/20 HCl, whereas the cysts were unharmed. The neutralized and washed sediment containing the cysts was sown into the part-liquid and part-solid medium to which starch and a suitable mixture of bacteria (strain La.), isolated originally from an excellent culture of *E. aulastomi*, had been added. Although in the first few experiments good hatches were obtained, as reported previously, more extensive work showed the results to be very uneven, and many poor hatches or complete failures resulted.

At Mr Dobell's suggestion flavine solution was tried. Cultures containing a rich growth of amoebae and no cysts (Bishop, 1932) were sedimented in 0·5 per cent NaCl solution and the sediment treated with 0·1 per cent flavine for 1 hour at room temperature. The sediment was subsequently freed from the flavine by repeated washing and resedimentation in 0·5 per cent salt solution. On examination all the amoebae were dead. A culture, in part-liquid part-solid medium inoculated with starch and the La. strain of bacteria, was made from the washed sediment but no amoebae grew. This experiment was repeated 6 times, but no growth of amoebae occurred. It was concluded that the action of 0·1 per cent flavine for 1 hour at room temperature is lethal

to the amoeboid form of *E. aulastomi*. Of 2 cultures which had been treated with 0·1 per cent flavine for half an hour at room temperature 1 was negative and the other showed a very poor growth of amoebae after 5 days. When 0·1 per cent flavine was used for shorter periods a few amoebae frequently survived.

Cultures containing a mixture of cysts and amoebae were therefore treated with 0·1 per cent flavine for an hour at room temperature. The cysts appeared uninjured and hatched afterwards. The hatches obtained from cysts treated thus were, on the whole, much better than from those treated with *N*/20 HCl for 10 min., but they were not really very satisfactory. Hatching experiments were performed in a variety of media including the liquid medium alone, a medium consisting of an inspissated whole-egg slope covered with the liquid medium, and a medium consisting of an inspissated horse-serum slope covered with egg-white in 0·5 per cent NaCl. To all these media rice starch was added. In the two latter media hatching did not occur, and in the first the hatch was much poorer than in the medium consisting of a horse-serum slope covered with inactivated horse-serum diluted 1 : 10 in 0·5 per cent NaCl. A much larger percentage of cysts hatched when cysts from those cultures where encystment had been almost universal were merely washed in 0·5 per cent NaCl and inoculated, without other treatment, into the last-mentioned medium to which rice starch had been added. Under such conditions 40–80 per cent of the cysts hatched, whereas of cysts treated with 0·1 per cent flavine from 20 to 40 per cent hatched.

Whereas the main details of excystation have been studied on material in which all free amoebae were known to have been killed, either by 0·1 per cent flavine or by *N*/20 HCl, the results have been supplemented by the more ample material obtained from untreated cysts from cultures containing very rich crops. Repeated observations upon cultures in which excystation was occurring threw no light upon the factors governing the excystation of individual cysts. Thus of a large group of cysts, all 4-nucleate, and in all of which the chromatoids had been absorbed, 4 or 5 individuals might hatch and the rest perish. Yet all had received, presumably, identical treatment.

Preparations for the study of excystation were fixed with Schaudinn's solution and stained with iron haematoxylin.

III. EXCYSTATION

A description of the precystic form of *E. aulastomi*, and of its cysts and the division of their nuclei, has already been published (Bishop, 1932). In that paper I stated that in the majority of the quadrinucleate cysts the nuclei were equal in size, but that occasionally cysts containing 1 large and 3 small nuclei were found. Since then the study of a much larger number of mature cysts has shown that whereas the majority have 4 nuclei of equal size, a small proportion have nuclei of the following kinds: 3 large and 1 small, 1 large

and 3 small, 2 large and 2 small, whilst cysts containing 1 large and 4 small, 3 large and 2 small, and 6 or 8 nuclei of equal size are occasionally met with.

In order to discover the proportion of such cysts to the normal ones, 4 batches of cysts, each from a different culture, were stained with Lugol's iodine solution. In the first batch 194 cysts were examined. Of these 180 (92·7 per cent) had 4 nuclei of equal size; 1 had 1 large nucleus and 4 small (0·51 per cent); 5 (2·57 per cent) had 2 large and 2 small nuclei; 4 (2·06 per cent) had 3 large and 1 small; 2 (1·03 per cent) had 1 large and 3 small; 1 (0·51 per cent) had 3 large and 2 small; and 1 (0·51 per cent) had 6 nuclei of the same size. In the second batch 334 cysts were examined. Of these 316 (94·61 per cent) had 4 nuclei of the same size; 3 (0·89 per cent) had 2 large and 2 small; 3 (0·89 per cent) had 3 large and 1 small; 11 (3·29 per cent) had 1 large and 3 small; and 1 (0·29 per cent) had 8 nuclei of equal size. In the third batch 120 cysts were examined and of these 115 (95·83 per cent) had 4 nuclei of equal size; 1 (0·83 per cent) had 2 large and 2 small nuclei; and 4 (3·33 per cent) had 1 large and 3 small nuclei. In the fourth batch 205 cysts were examined, and of these 196 (95·6 per cent) had 4 nuclei of equal size; 2 (0·97 per cent) had 2 large and 2 small nuclei; 1 (0·48 per cent had 3 large and 1 small; 5 (2·48 per cent) had 1 large and 3 small; and 1 had 8 nuclei of equal size.

Excystation does not occur until the glycogen and the chromatoids are absorbed when the cysts may be termed "ripe". The 4 nuclei are visible in the unstained living cyst. Dobell (1928) found that immature cysts of *E. histolytica* ceased to develop when cooled to room temperature, and that if kept long at this temperature they were unable to continue their development when reincubated. Since development of immature cysts ceased at room temperature he was able to estimate the time taken in the ripening process of the 4-nucleate mature cysts. Unfortunately at all temperatures at which the 4-nucleate cyst of *E. aulastomi* will remain healthy the immature cysts (uni- and binucleate) continue their development. It has therefore been quite impossible to arrive, within any degree of accuracy, at the time taken for the absorption of the glycogen and chromatoids. The glycogen is absorbed much more rapidly than the chromatoids. The time varies considerably with the temperature to which the cysts are subjected. Thus of cysts stored at 2°C. many had lost their glycogen in 3 days but none had lost their chromatoids, and many still possessed these after 10–15 days, though at the end of 20 days they had disappeared in almost all. At room temperature (22–24°C.) these structures had disappeared in most cysts in from 3 to 7 days.

When cysts which had been freed from accompanying amoebae by treatment with 0·1 per cent. flavine or *N*/20 HCl and which still contained glycogen and chromatoids were inoculated into part-liquid part-solid medium with starch and bacteria, excystation began about 48 hours after inoculation. After 72 hours no further hatching occurred, and those cysts which had not hatched

died. But when "ripe" cysts were inoculated into such medium excystation sometimes began within 3–4 hours.

When excystation is about to take place the protoplasm shrinks away from the cyst wall so that it lies, a small, dense sphere with the 4 nuclei close together in its centre (Fig. 1), freely in the cyst. At this stage it frequently fills only about two-thirds of the cyst. A few vacuoles appear in the protoplasm. The protoplasm becomes more fluid and small, stiff, slow-flowing pseudopodia are formed. The amoeboid mass moves slowly round and round inside the cyst wall, the pseudopodia from time to time appearing to press against it as if testing it. In many cysts this movement may continue for an hour to 2 hours. Suddenly, as in *E. histolytica* (Dobell, 1928), the tip of one of the pseudopodia passes through a perforation in the cyst wall and bulges out on the outer side of the cyst. The perforation is extremely small. Usually about one-third of the body volume passes outside and is then slowly drawn in again and the flowing, rotatory movement continues. In *E. histolytica* Dobell states that on withdrawal a little bead of cytoplasm remains on the outside and then the flow is repeated. But in *E. aulastomi* no such bead is seen, and though the outward flow may be repeated almost immediately as long as half an hour may relapse before it is resumed. In the meantime the perforation is invisible and it is therefore impossible to be certain whether the second flow is through the same opening as the first. This outward and inward flow may occur 5 or 6 times, separated by 10–30 min. intervals, each succeeding interval being shorter than the preceding one, until finally the whole amoeba flows slowly through the minute pore. Each of the 4 nuclei becomes very compressed as it passes through the tiny opening, but they become spherical again as soon as they get outside. The total time taken by excystation varied from 1 to 4 hours. Little or no residue is left in the cyst. The empty cyst remains intact for many hours and frequently becomes invaded by bacteria though, as in *E. histolytica* (Dobell, 1928), the opening is invisible.

IV. THE METACYSTIC DEVELOPMENT

In the following description of the metacystic development the terminology introduced by Dobell (1928) in his study of *E. histolytica* will be used.

The newly excysted amoeba is devoid of cytoplasmic inclusions. It measures 8–10μ (Fig. 2). It flows about ingesting bacteria and starch and increases in size (Fig. 3) so that it soon measures 12–13μ. The 4 cystic nuclei also grow larger. They lie close together, but owing to the ingested food they soon become obscured in the living organism and their subsequent development has been studied on fixed and stained material. In cultures where excystation is just beginning only amoebae with 4 cystic nuclei are found, but a little later, in addition to these amoebae with 3 large cystic nuclei and 2 small daughter nuclei (*NNNnn*, Fig. 19), which have arisen by the division of the fourth cystic nucleus, appear. The nuclear division (Fig. 4) producing this 5-nucleate amoeba is commonly found. At the time of this division the

nuclei separate, but if no cytoplasmic division follows they may run together again (Fig. 19). The separation of the nuclei at the time of nuclear division occurs at all stages of the metacystic development. In many individuals a cytoplasmic division occurs (Fig. 10) giving rise to a large amoeba (quadrinucleate heptagen, *NNNn*) with 3 large cystic nuclei and 1 daughter nucleus and an amoebula with 1 daughter nucleus. In the quadrinucleate heptagen the nuclei all come together again. It is possible that not all amoebae containing 3 cystic nuclei and 1 daughter nucleus have arisen in this way as a small percentage (p. 59) may have arisen directly by the excystation of cysts containing 3 large nuclei and 1 small; but excystation of such cysts has never been seen. Not all amoebae with 3 cystic nuclei and 2 daughter nuclei divide in this way, as a type of cytoplasmic division more frequently met with is into 2 amoebae of almost equal size (Fig. 26), one containing 2 large cystic nuclei and 1 daughter nucleus (Fig. 27, trinucleate pentagen, *NNn*) and the other 1 large cystic nucleus and 1 daughter nucleus (Fig. 23, binucleate trigen, *Nn*). When cytoplasmic division is complete, the nuclei in each amoeba are found close together. Amoebae bearing such nuclear combinations are very common. The study of cultures containing more advanced stages of excystation has shown that many quinquenucleate octogens (*NNNnn*) undergo further nuclear division before cytoplasmic division occurs. The third cystic nucleus may divide (Fig. 20) to give rise to a 6-nucleate octogen (Fig. 21, *NNnnnn*). One (Fig. 5) or both (Fig. 22) of these cystic nuclei may divide again giving rise in the first case to a septemnucleate octogen (Fig. 6) and in the second to an amoeba with 8 daughter nuclei (Fig. 7). The remaining cystic nucleus in the septemnucleate octogen (Fig. 8) may subsequently divide. These 7- and 8-nucleate amoebae are not very common and it has been impossible to trace their later development. Not all the 8-nucleate amoebae may have arisen by the nuclear division of a 4-nucleate excysted amoeba, for a few (p. 60) may be the result of the excystation of 8-nucleate cysts.

Amoebae with 2 cystic nuclei and 3 daughter nuclei (quinquenucleate heptagens, *NNnnn*) are frequent. The division of the third cystic nucleus in a quadrinucleate heptagen (*NNNn*) produces such a form. They may divide (Fig. 14) by bipartition of the cytoplasm into a trinucleate tetragen (*Nnn*, Fig. 45) and a binucleate trigen (*Nn*, Fig. 23). Trinucleate tetragens (*Nnn*) are common. They may, by division of the cystic nucleus (Fig. 36), give rise to an amoeba with 4 daughter nuclei (*nnnn*). This amoeba may then divide into 2 amoebae each containing 2 daughter nuclei (*nn*, Fig. 32). Each of these binucleate amoebae presumably produces 2 uninucleate amoebulae, but these cytoplasmic divisions have not been recognized with certainty. An alternative method of development of trinucleate tetragens (*Nnn*) is by the division of the cytoplasm into 2 amoeba (Fig. 17), a uninucleate digen (*N*) and an amoeba with 2 daughter nuclei (Fig. 32), without any previous division of the nucleus. Presumably the amoeba containing 1 cystic nucleus will, by nuclear and cytoplasmic division, give rise to 2 amoebulae, but such divisions would be indis-

tinguishable from normal divisions. Binucleate trigens (*Nn*) are very common. Not only do they arise by cytoplasmic division of the quinquenucleate heptagens (Fig. 14) but also from quinquenucleate octogens (Fig. 26). By division of the cystic nucleus a trinucleate trigen (*nnn*, Fig. 28) may be produced. The future development of such amoebae has not been seen. It is possible (Fig. 25) that binucleate trigens may, without further nuclear division, undergo bipartition of the cytoplasm to form an amoeba containing a single cystic nucleus and a uninucleate amoebula; but only one individual undergoing such a division has been seen.

Not all quinquenucleate heptagens (*NNnnn*) undergo cytoplasmic division after division of the third cystic nucleus, for individuals in which the second cystic nucleus is dividing (Figs. 9 and 12) are occasionally found. Presumably amoebae containing 1 cystic nucleus and 5 daughter nuclei (*Nnnnnn*, Fig. 13) have been produced in this way. Rare amoebae (Fig. 18) containing 7 daughter nuclei have probably arisen by the division of the remaining cystic nucleus. Their further development has not been traced.

Trinucleate pentagens (*NNn*, Fig. 27) arise, as described above, by the cytoplasmic division (Fig. 26) of the quinquenucleate octogens. They are very common and several methods of their future development have been seen. By direct cytoplasmic division, without further nuclear division, they may give rise (Fig. 24) to a uninucleate digen (*N*) and a binucleate trigen (*Nn*, Fig. 23). The future development of such amoebae, produced by other methods, has already been discussed. One of the cystic nuclei of a trinucleate pentagen may, before any cytoplasmic division occurs, divide (Fig. 31) and produce a quadrinucleate pentagen (Fig. 30, *Nnnn*). By bipartition of the cytoplasm (Fig. 38) this then may produce a binucleate trigen (*Nn*) and a binucleate digen (*nn*). By a different type of cytoplasmic division (Fig. 37) a trinucleate tetragen and a uninucleate amoebula may be produced. The subsequent development of trinucleate tetragens has been discussed previously. One quadrinucleate pentagen was seen (Fig. 34) in which the remaining cystic nucleus was in the telophase. Whether an amoeba containing 5 daughter nuclei would be produced, or whether cytoplasmic division would take place immediately is not known, but amoebae containing 5 daughter nuclei are found though rarely. Their future development has not been seen.

Individuals with 3 large nuclei (Fig. 15) are not rare. At first it was concluded that they arose by the delayed cytoplasmic division and the growth of the nuclei of trigens (*nnn*). Stages in division of 1 of the nuclei (Figs. 39 and 40) of such amoebae make this interpretation untenable. The most probable explanation is that the excysted amoeba (quadrinucleate octogen, *NNNN*), before any nuclear division occurs, may undergo cytoplasmic fission to produce a uninucleate digen (*N*) and a trinucleate hexagen (*NNN*). Such a cytoplasmic division has never been seen with certainty. Though Fig. 16 strongly suggests such a division it is possible that this individual is a tetragen (*nnnn*) in which cytoplasmic division has been delayed and the nuclei have

become abnormally large. However carefully excystation experiments are devised, the cysts hatch at various times during a long period, so that a culture soon contains many different stages. In early preparations, however, a few trinucleate amoebae and amoebae with 1 large nucleus have been found in addition to quadrinucleate octogens and quinquenucleate octogens (*NNNnn*). They may have arisen by division of quinquenucleate octogens into trinucleate hexagens and binucleate digens, but these latter amoebae have not been found in the earliest stages of excystation. It is difficult to imagine how they have arisen if not by a cytoplasmic division of a quadrinucleate octogen. They are very rare in young cultures (8–24 hours), but later (24–48 hours) the proportion of such trinucleate amoebae increases. After division of one of the cystic nuclei (Figs. 39 and 40) 2 binucleate trigens may be produced by bipartition (Fig. 35) of the body.

Amoebae containing 2 large nuclei (*NN*, binucleate tetragens) are fairly frequent. A small proportion of binucleate amoebae is found in ordinary cultures of *E. aulastomi* where excystation is not taking place. In such amoebae (Bishop, 1932, Plate XI, fig. 12) the nuclei lie well apart. In binucleate amoebae in cultures where excystation is taking place the nuclei lie close together (Fig. 42). Division of one of the nuclei in binucleate amoebae occurring in non-excysting cultures has never been seen, but in binucleate amoebae in cultures where excystation is taking place division of one of the nuclei (Fig. 47) to form a trinucleate tetragen (Fig. 45) is fairly frequent. Normally bipartition of the cytoplasm (Fig. 50) to form a binucleate trigen (*Nn*) and a uninucleate amoebula then takes place, but a single specimen (Fig. 46) suggested a possible tripartition. This is the only individual in all the material which suggests a tripartition.

A few amoebae containing more than 4 cystic nuclei or their division products have been found (Figs. 51–54). It is probable that these have arisen by the excystation of 6- or 8-nucleate cysts. They are so rare that it has been impossible to follow their development.

Since cysts do not hatch simultaneously the presence at one time of many different stages makes the interpretation of the findings very difficult. The difference in size in the nuclei, both cystic and daughter nuclei, in different individuals at the same, or almost the same, stage of metacystic development (see figures), also adds considerably to the difficulties. Growth of the daughter nuclei may also take place between succeeding divisions. Though many differen stages of development may occur in one preparation, the greater proportion are resting stages with the cystic and daughter nuclei grouped together. Amoebae containing nuclei undergoing division form only a very small percentage of the metacystic amoebae, while individuals undergoing cytoplasmic division are even scarcer; it is upon these stages that the interpretation of the whole development depends.

It has been impossible to estimate, with any degree of accuracy, the time taken to complete the metacystic development of the product of any indi-

vidual cyst; but it is clear that, as in *E. histolytica*, every cyst must sooner or later produce 8 amoebulae.

V. DISCUSSION

Throughout the metacystic development of *E. aulastomi* there is no indication of any sexual process. The development proceeds by a sequence of nuclear and cytoplasmic divisions. In this it resembles the metacystic development of *E. histolytica* as described by Dobell (1928) and later by Cleveland & Sanders (1930).

When excystation occurs in cultures of *E. aulastomi* a 4-nucleate amoeba in which all the nuclei are in juxtaposition is liberated. A 4-nucleate amoeba hatches from the cyst of *E. histolytica* both *in vivo* (Chatton, 1917; Tanabe, 1934) and in culture media (Dobell, 1928; Cleveland & Sanders, 1930). Brug (1928) saw 4-nucleate amoebae in a culture of *E. histolytica* in which excystation was taking place. In *E. terrapinae* (Sanders & Cleveland, 1930) and *E. ranarum* (Sanders, 1931), both being species in which the mature cyst is 4-nucleate, a 4-nucleate amoeba also hatches from each cyst.

As in *E. histolytica* (Dobell, 1928) cysts of *E. aulastomi* do not hatch until the chromatoids and glycogen are absorbed. Hatching of uninucleate and binucleate cysts was never seen in either living cysts of *E. aulastomi* or in fixed and stained preparations. Neither Dobell nor Cleveland & Sanders describe the excystation of such cysts in *E. histolytica*, but Hegner, Johnson & Stabler (1932) and Tanabe (1934) describe and figure their excystation. According to their descriptions, excystation of these cysts did not take place through a small pore as in *E. histolytica* (Dobell, 1928; Cleveland & Sanders, 1930), but through a large opening. To me their illustrations suggest artificial rupture of the cyst wall. A large opening in the cyst wall was never seen in *E. aulastomi*.

According to Dobell (1928) the excysted quadrinucleate amoeba of *E. histolytica* ultimately gives rise, by a series of nuclear and cytoplasmic divisions, to 8 uninuclear amoebulae. Every division of the cytoplasm is preceded by a nuclear division with the exception of the division of the cytoplasm of binucleate digens formed in certain ways (Dobell, 1928, Text-fig. A). As a result of these interpolating cytoplasmic divisions, amoebae with 6, 7 or 8 daughter nuclei do not arise in the course of normal development. Cleveland & Sanders (1930) state that in their cultures of excysting *E. histolytica* cytoplasmic division may occur after each nuclear division, or it may be delayed so that amoebae containing 6, 7 or 8 daughter nuclei are produced. They failed, however, as I failed in my study of such amoebae in the metacystic development of *E. aulastomi*, to observe cytoplasmic division in such multinucleate amoebae. Though Cleveland & Sanders give a detailed scheme of the possibilities and probabilities of the metacystic development of *E. histolytica* their description is not fully supported, as is Dobell's, by illustrations of the actual nuclear and cytoplasmic divisions on which their interpretations are based.

In *E. aulastomi* cytoplasmic division does not necessarily follow after each nuclear division.

Both Dobell, and Cleveland & Sanders describe tripartition and quadripartition of the cytoplasm. Such multiple cytoplasmic divisions are not found in *E. aulastomi*. Only one probable tripartition was seen. Owing to the absence of these multiple cytoplasmic divisions and to the frequency of nuclear divisions succeeding each other without any interpolating cytoplasmic divisions, amoebae containing combinations of cystic and metacystic nuclei are found in *E. aulastomi* which are absent or very rare in *E. histolytica*. A comparison of Dobell's plates with mine will illustrate this. Dobell never saw trinucleate hexagens in *E. histolytica*, but these are not uncommon in *E. aulastomi*; for whereas in *E. histolytica* cytoplasmic division of the excysted amoeba never occurs before the first nuclear division, it is highly probable (p. 63) that in *E. aulastomi* a division into a uninucleate digen (*N*) and a trinucleate hexagen (*NNN*) sometimes takes place, though the actual division has not been seen with certainty. A division of the quadrinucleate octogen into 2 binucleate tetragens (*NN*) has never been seen in *E. aulastomi*, but binucleate amoebae with 2 cystic nuclei are fairly common. In *E. histolytica* Dobell states that they are very rare.

Sanders (1931) believes that the metacystic development of *E. ranarum* is similar to that of *E. histolytica*. The few stages which she figures certainly fit into the scheme of metacystic development devised by Cleveland and herself, but she presents no figures of the actual cytoplasmic and nuclear divisions which are essentially the foundation of all such interpretations.

The metacystic development of *E. terrapinae* (Sanders & Cleveland, 1930) differs from that of *E. histolytica* in that the excysted quadrinucleate amoeba "gives rise by cytoplasmic division to two binucleate amoebae, which, by cytoplasmic division produce four uninucleate amoebae". No nuclear division is therefore involved and the final product is 4 uninucleate amoebulae instead of 8 as in *E. histolytica*.

VI. Summary

1. In *Entamoeba aulastomi* excystation does not occur *in vitro* until the glycogen and chromatoids have been absorbed.

2. The product of excystation is a quadrinucleate amoeba.

3. The amoeba escapes through a minute pore in the cyst wall.

4. No evidence of sexual phenomena has been obtained during the metacystic cycle.

5. The excysted quadrinucleate amoeba undergoes a series of nuclear and cytoplasmic divisions. These divisions do not necessarily synchronize or alternate with one another, but by them 8 uninucleate amoebulae containing daughter nuclei are ultimately produced from every cyst. Amoebae containing 6, 7 or 8 nuclei may appear in the course of the metacystic development.

I wish to thank Mr Clifford Dobell, F.R.S., for the advice he has given me throughout this study and for kindly reading through the manuscript.

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EXPLANATION OF PLATES

All figures are drawn with an Abbé camera lucida under a 1/12 in. fluorite oil-immersion lens (Leitz), with a × 20 periplanatic eyepiece, at an approximate magnification of 3300 diameters. The figures have been reduced to 3/4 in reproduction and their present magnification is 2475 diameters. All the figures are of *Entamoeba aulastomi* grown in culture.

PLATE II

Fig. 1. Cyst containing 4-nucleate amoeba.

Fig. 2. Newly excysted amoeba (quadrinucleate octogen).

Fig. 3. Older excysted 4-nucleate amoeba which has ingested food and increased in size.

Fig. 4. Stage in nuclear division in quadrinucleate octogen (*NNNN*) to form a quinquenucleate octogen (*NNNnn*) or, by later division of the cytoplasm, a trinucleate pentagen (*NNn*) and a binucleate trigen (*Nn*).

Fig. 5. Stage in nuclear division in sexnucleate octogen (*NNnnnn*) to form, unless cytoplasmic division takes place, a septemnucleate octogen (*Nnnnnnn*).

Fig. 6. Septemnucleate octogen (*Nnnnnnn*).

Fig. 7. Octonucleate octogen.

Fig. 8. Septemnucleate octogen (*Nnnnnnn*) in which the remaining cystic nucleus is preparing to divide to form, unless cytoplasmic division occurs, an octogen.

Fig. 9. Quinquenucleate heptagen (*NNnnn*) in which 1 of the cystic nuclei is dividing to produce a sexnucleate heptagen (*Nnnnnn*). This amoeba may undergo further cytoplasmic division.

Fig. 10. Quinquenucleate octogen in which the body is dividing to produce a quadrinucleate heptagen (*NNNn*) and a uninucleate amoebula (*n*).
Fig. 11. Quadrinucleate heptagen (*NNNn*).
Fig. 12. As Fig. 9 but a later stage in mitosis.
Fig. 13. Sexnucleate heptagen (*Nnnnnn*).
Fig. 14. Quinquenucleate heptagen (*NNnnn*) undergoing cytoplasmic division to produce a binucleate trigen (*Nn*) and a trinucleate tetragen (*Nnn*).
Fig. 15. Trinucleate amoeba (*NNN*?).
Fig. 16. Quadrinucleate amoeba (*NNNN*?) dividing to produce a trinucleate (*NNN*?) and a uninucleate (*N*?).
Fig. 17. Trinucleate tetragen (*Nnn*) dividing to produce a uninucleate digen (*N*) and a binucleate digen (*nn*).
Fig. 18. Amoeba containing 7 nuclei.
Fig. 19. Quinquenucleate octogen (*NNNnn*).
Fig. 20. Quinquenucleate octogen (*NNNnn*) in which 1 of the cystic nuclei is just completing its division to produce a sexnucleate octogen (*NNnnnn*) unless cytoplasmic division occurs immediately.
Fig. 21. Sexnucleate octogen (*NNnnnn*).
Fig. 22. Sexnucleate octogen (*NNnnnn*) in which 2 cystic nuclei are dividing to produce, unless cytoplasmic division takes place, an 8-nucleate amoeba (*nnnnnnnn*).
Fig. 23. Binucleate trigen (*Nn*).
Fig. 24. Trinucleate pentagen (*NNn*) dividing to produce a binucleate trigen (*Nn*) and a uninucleate digen (*N*).
Fig. 25. Binucleate trigen dividing to form a uninucleate digen (*N*) and a uninucleate amoebula (*n*).
Fig. 26. Quinquenucleate octogen (*NNNnn*) dividing to form a binucleate trigen (*Nn*) and a trinucleate pentagen (*NNn*).
Fig. 27. Trinucleate pentagen (*NNn*).
Fig. 28. Binucleate trigen (*Nn*) in which the cystic nucleus is dividing to form a trigen (*nnn*).
Fig. 29. Trinucleate trigen (*nnn*).
Fig. 30. Quadrinucleate pentagen (*Nnnn*).
Fig. 31. Trinucleate pentagen in which 1 cystic nucleus is dividing to form, unless cytoplasmic division also takes place, a quadrinucleate pentagen (*Nnnn*).
Fig. 32. Binucleate digen (*nn*).
Fig. 33. 5-nucleate amoeba (*nnnnn*).
Fig. 34. Quadrinucleate pentagen (*Nnnn*) in which the remaining cystic nucleus is dividing to produce, unless cytoplasmic division occurs, a quinquenucleate pentagen (*nnnnn*).
Fig. 35. Trinucleate hexagen (*NNN*) in which 1 cystic nucleus has divided and the body is dividing to produce 2 binucleate trigens (*Nn*).
Fig. 36. Trinucleate tetragen (*Nnn*) in which the cystic nucleus is dividing to produce a quadrinucleate tetragen (*nnnn*).
Fig. 37. Quadrinucleate heptagen (*Nnnn*) which is dividing to produce a uninucleate amoebula (*n*) and a trinucleate tetragen (*Nnn*).
Fig. 38. Trinucleate pentagen (*NNn*) in which 1 cystic nucleus has just divided. The body is beginning to divide to produce a binucleate digen (*nn*) and a binucleate trigen (*Nn*).

PLATE III

Fig. 39. Trinucleate hexagen (*NNN*) in which 1 cystic nucleus is dividing to produce, if cytoplasmic division does not take place, a quadrinucleate hexagen (*NNnn*).
Fig. 40. As Fig. 39. Later stage of mitosis.
Fig. 41. Quadrinucleate hexagen (*NNnn*).
Fig. 42. Binucleate tetragen (*NN*).
Fig. 43. Quadrinucleate hexagen (*NNnn*) in which 1 cystic nucleus is dividing to form, if cytoplasmic division does not take place, a quinquenucleate hexagen (*Nnnnn*).
Fig. 44. Quinquenucleate hexagen (*Nnnnn*).

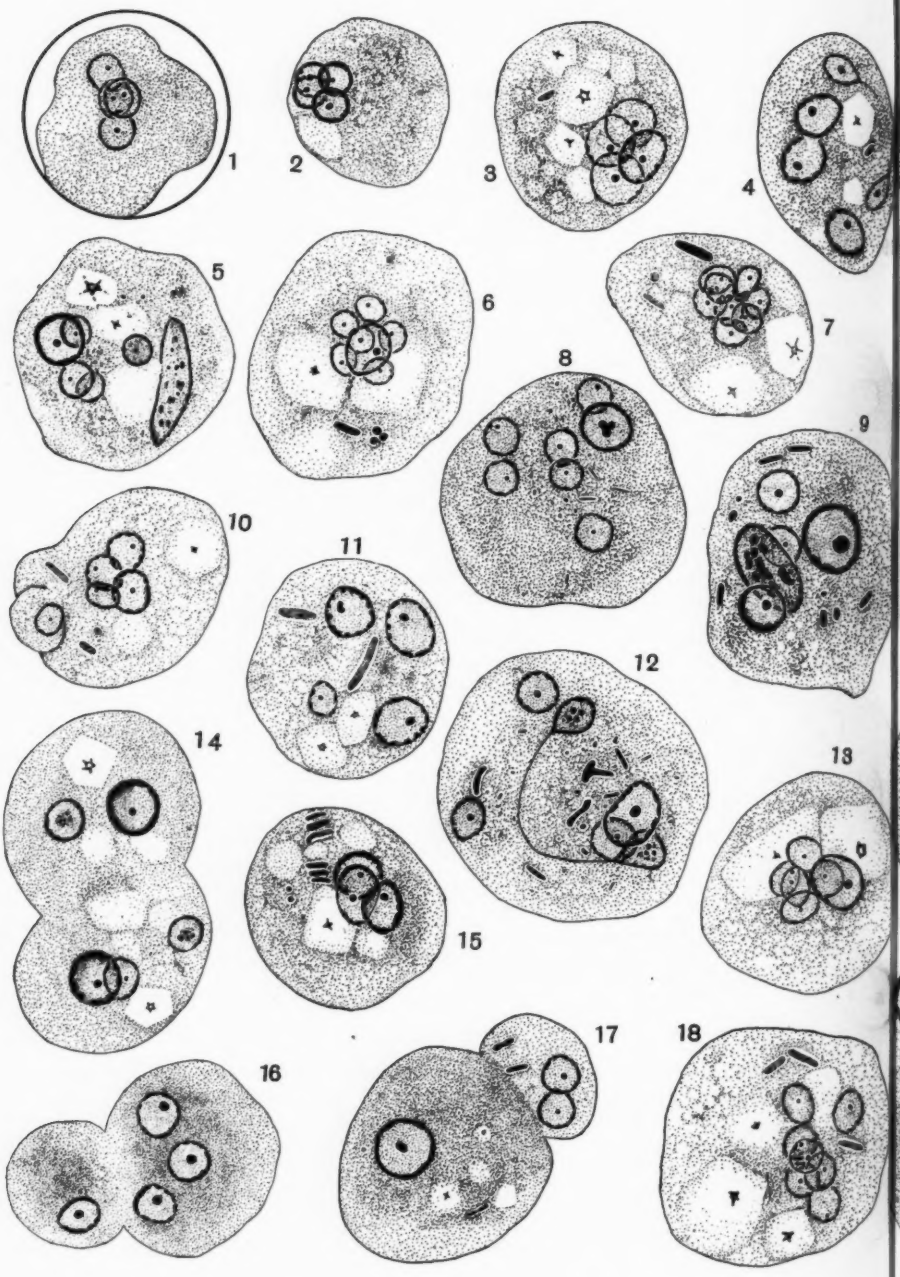

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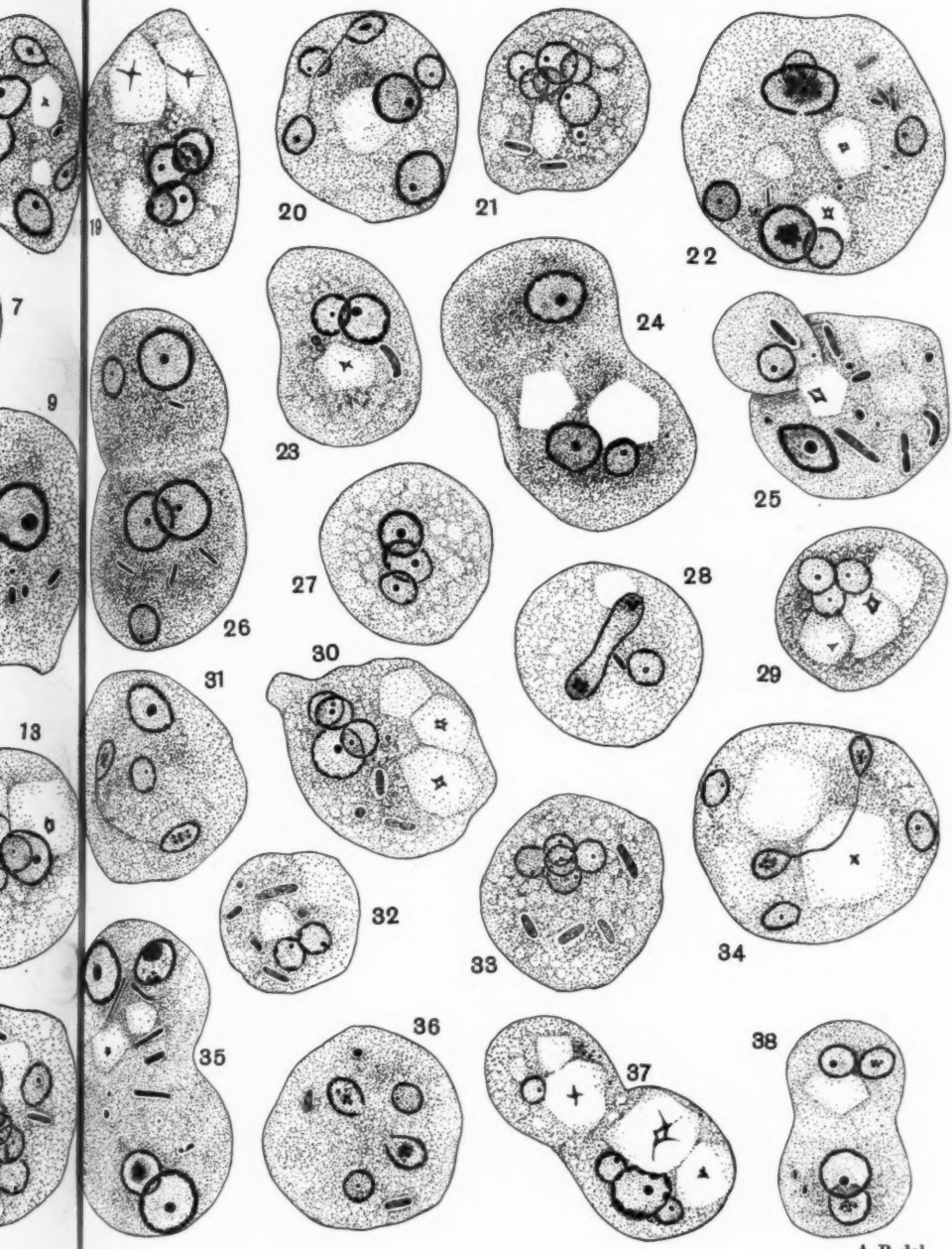
PLATE II



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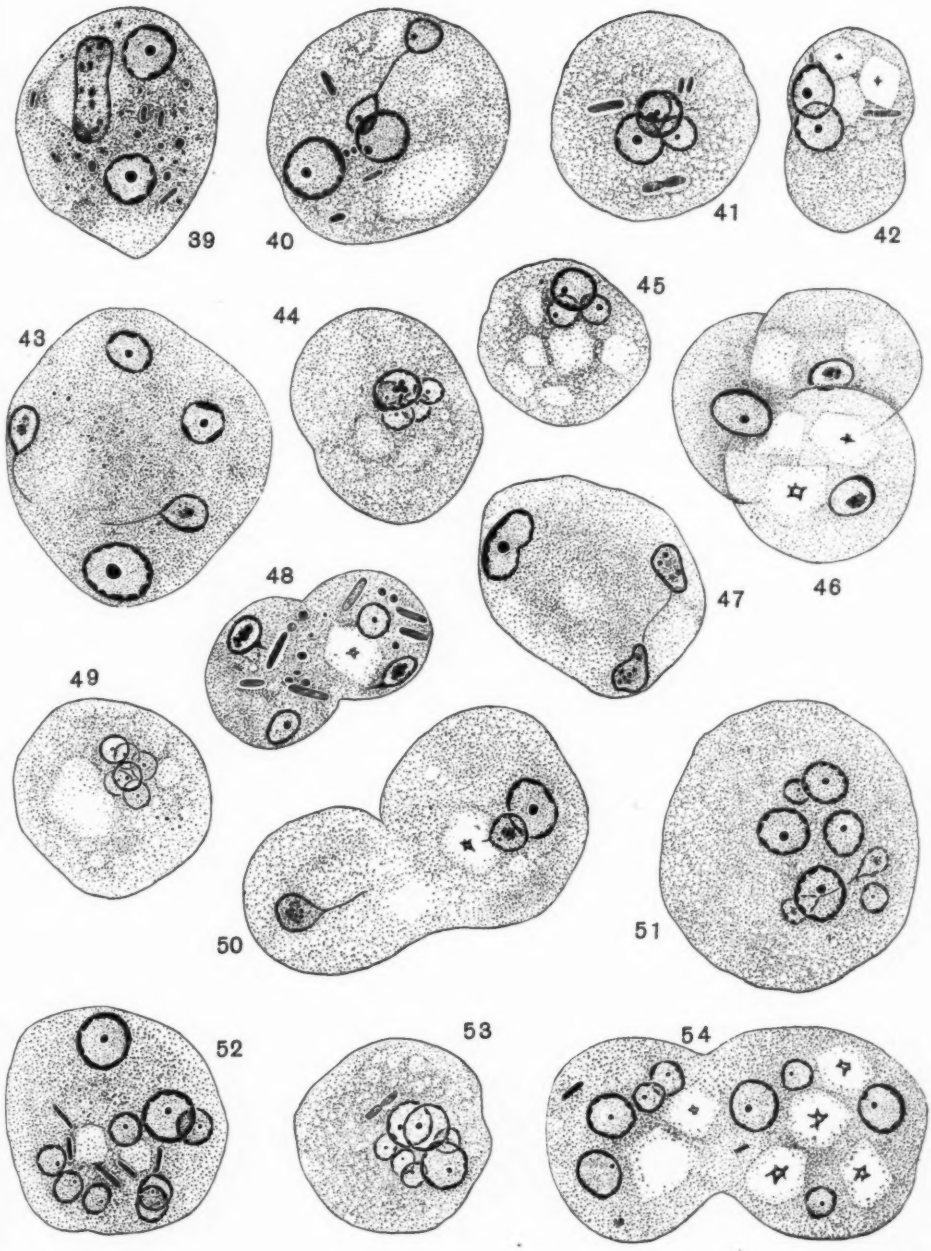

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Fig. 45. Trinucleate tetragen (*Nnn*).
Fig. 46. Tripartition (?) of trinucleate amoeba (*Nnn*).
Fig. 47. Binucleate tetragen (*NN*) in which 1 cystic nucleus is dividing to form, if cytoplasmic division does not take place, a trinucleate tetragen (*Nnn*).
Fig. 48. Trinucleate tetragen (*Nnn*) in which the cystic nucleus has just divided. The body is dividing to form 2 binucleate digens (*nn*).
Fig. 49. 6-nucleate amoeba.
Fig. 50. Binucleate tetragen (*NN*) in which 1 cystic nucleus has divided. The body is dividing to form a uninucleate amoebula (*n*) and a binucleate trigen (*Nn*).
Fig. 51. Amoeba with 5 cystic nuclei, of which 1 is dividing, and 2 daughter nuclei.
Fig. 52. Amoeba with 2 cystic nuclei and 7 daughter nuclei.
Fig. 53. Amoeba with 3 cystic nuclei and 4 daughter nuclei.
Fig. 54. Amoeba with 4 cystic nuclei and 4 daughter nuclei. The body is dividing to form 2 amoebae each containing 2 cystic nuclei and 2 daughter nuclei.

(*MS. received for publication* 24. IV. 1936.—Ed.)

STUDIES ON A VIRUS FOUND IN THE ROOTS OF CERTAIN NORMAL-LOOKING PLANTS

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(With Plates IV and V and 1 Figure in the Text)

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I. Introductory

In a previous paper (Smith & Bald, 1935) an account was given of a virus disease which in the absence of insect vectors developed in tobacco plants growing in sterile soil. Such conditions are usually considered proof against accidental virus infections with the exception of mechanical contamination. The disease developed *naturally* only on tobacco and *Nicotiana glutinosa*, and as the symptoms produced were solely necrotic, the name of "tobacco necrosis" was suggested for the disease.

From the beginning it was evident that the disease of tobacco necrosis differed in certain respects from other plant virus diseases. First, the virus never became systemic in the host plant and was usually confined to one pair of leaves, the first-developed and consequently the lowest leaves on the plant. Secondly, the symptoms of a natural infection always differed from those of an artificial inoculation; the former showed a necrosis of the midrib and larger veins, the latter consisted of numerous necrotic lesions scattered over the leaf

surface. Thirdly, as mentioned above, the disease developed under what were considered to be virus-proof conditions.

Another point which required explanation was the regular appearance of the disease in the ordinary stocks of White Burley tobacco plants from November to February of each year, followed by its complete disappearance until the next November. In these circumstances it was difficult to understand what was the source of the apparent new infection each autumn. This problem was solved by the discovery that a high percentage of the ordinary healthy tobacco plants contained the virus in their roots throughout the year and that it was only during the late autumn and winter that the infective agent ascended into the lower leaves and produced visible symptoms. The great majority of the tobacco plants containing this agent never develop any symptoms of disease but remain absolutely normal in appearance until maturity.

This discovery also explained the difference in the appearance of natural and artificial infections, since in the former instance the virus was ascending the stem from the roots and passing along the midribs of the two lowest leaves which it destroyed *en route*. In the latter case of artificial inoculation numerous lesions were produced on the leaves at the points of entry of the virus. In addition, a good explanation was furnished for the invariable appearance of the symptoms in the lowest leaves, since these would be the first encountered by the ascending virus, and this explanation rendered unnecessary any suggestion of contamination by contact with the soil which, being sterilized, was in any case an unlikely source of infection.

The questions that remain to be answered therefore are, from where does this apparent virus come and how does it reach the plants? In the present paper a number of experiments are described which were designed to investigate these problems.

II. EXPERIMENTAL METHODS

All experimental plants were grown in the insect-proof glasshouses at Cambridge. In certain experiments the plants were grown for additional precaution inside a frame of cellophane contained in the glasshouse.

The soil and sand used were sterilized in an electric heater of the box type. The temperature of the centre of the soil in the container usually reached 100° C. For some experiments the sand in which the plants were grown was baked in a metal container on a Primus stove and the soil was autoclaved. Other modifications in procedure are described in the accounts of the particular experiments concerned. In making inoculations from the roots of plants, the roots in question were thoroughly washed under the tap until all visible particles of soil were removed. They were then ground up in a sterile mortar and the resulting extract inoculated by means of ground-glass spatulae to the test plants. In the previous paper it was shown that the black-seeded cowpea (*Vigna sinensis*) was a valuable experimental plant, and in the first part of the work this was used. Later, however, it was discovered that the

French bean (*Phaseolus vulgaris*), var. Canadian Wonder, was even more sensitive to the virus and was used extensively in the latter part of the work. This plant develops very large numbers of dark red necrotic lesions within 48 hours of inoculation at temperatures of about 60–70° F. (Pl. IV, fig. 1).

III. Experiments demonstrating the presence of the virus in the roots of various species of normal-looking plants

In these experiments the roots of a large number of different plants were tested for the presence of the infective principle. There were two main objects in view, first, to find out what plants or types of plant were liable to contain the virus in the roots, and secondly what proportion of such plants actually contained the virus. The plants tested are briefly discussed under the headings of their families.

Solanaceae

Nicotiana tabacum, tobacco; several varieties.

As mentioned already it is only in tobacco and the allied species, *Nicotiana glutinosa*, that naturally occurring disease symptoms caused by this virus have been observed. These symptoms develop most commonly from November to February, and occasionally isolated plants of considerable size develop symptoms on one or both of the two lowermost leaves.

Out of a total of 244 healthy-looking tobacco plants tested, 166, or 68 per cent, contained the infective principle in the roots. It should be pointed out that none of these tobacco plants at any time showed symptoms of disease or was other than normal in appearance. The tobacco plants used in these tests were derived from seed which came from various sources; the White Burley plants were grown from seed obtained from plants grown in the insect-proof glasshouses and from seed from Portugal. Other varieties such as Virginia and Kentucky White Stem were obtained from dealers, whilst one unnamed variety came from Southern Rhodesia.

Nicotiana langsdorffii.

The roots of 11 plants of this species were tested, 1 of which gave a positive virus reaction on cowpeas.

Tomatoes.

The roots of 33 tomato plants of different sizes were tested; of these 15 contained the virus in the roots. Tomato plants have never been observed to show any visible signs of natural infection with this virus during the 9 years they have been grown in the glasshouses in Cambridge. If inoculated artificially, however, local necrotic lesions develop on the leaves without any further spread of infection.

Solanum nigrum, Black nightshade.

One plant out of 6 of this species tested contained the virus in the roots.

Primulaceae

In order to discover whether the infective principle was present in the roots of plants of a different species from tobacco or the other members of the Solanaceae, the experiments were extended to include various other families. It was found that the roots of normal-looking *Polyanthus* plants which had been grown from seed in the glasshouse contained the virus in quite large quantities. Out of 12 plants tested, 6 gave a positive reaction. Similarly with *Primula obconica*, out of 7 plants tested, 5 contained the virus in the roots.

Compositae

Tests were made of the roots of some small aster plants growing in the glasshouse. Out of 6 plants tested 2 gave a positive virus reaction on French beans.

Geraniaceae

Inoculations were made from the roots of a number of plants of the Zonal Pelargonium. Some of these were grown originally from seed in the glasshouse but had been put out into a cold frame. Three of the plants were derived from cuttings taken from pelargoniums from outside sources. Out of 10 plants tested 6 gave a positive virus reaction on French beans.

Leguminosae

The virus has also been found in the roots of absolutely normal-looking plants of French beans. This is remarkable in view of the fact that the French bean is excessively sensitive to artificial inoculation with the virus and has been used extensively as a test plant in this work. The leaf tissue of the French bean reacts to the presence of a very small trace of the virus, while death of the plant seems invariably to follow if the virus enters the stem or petiole. Yet in spite of this the infective agent is present in the secondary roots of quite a large number of French beans. It is extremely difficult to understand how the virus can have entered into the roots of this plant without producing any sign of its presence. French beans showing symptoms of the disease by natural infection have never been observed.

IV. EXPERIMENTS TO INVESTIGATE THE POSSIBILITY OF VIRUS TRANSMISSION THROUGH THE SEED

Investigation of the possible transmission of the virus through the seed is of course an important part of these studies, and a number of experiments were carried out in the hope of solving this problem.

In the first experiment the ripe seed capsules of 10 White Burley tobacco plants were selected and the contents ground up and inoculated separately, each to a pot of French beans. Tests of the plants from which the seed was

taken showed the virus to be present in the roots of 6 out of the 10 plants. All these inoculations with the ripe seed gave negative results.

In the next experiment 12 flower heads and 12 green seed capsules were gathered from 12 mature plants of White Burley tobacco growing in the field, which had been selected because of the presence of the virus in the roots. Three cowpeas were inoculated with the ground-up tissue of each flower and 3 with each green seed capsule similarly treated. All these inoculations with the flower petals and the green capsules proved negative.

It may be suggested that the virus is actually present in the seed of tobacco and other plants but in form or quantity which is not detectable by the available methods of technique. If this be so, then by germinating the seeds on blotting paper it is reasonable to suppose that the virus would be enabled to multiply sufficiently in the roots of the resulting seedlings to give a positive reaction on inoculation to the test plants. This experiment was performed as follows. Seed of White Burley tobacco was germinated on damp blotting paper in Petri dishes. When the seedlings had developed 2 leaves and a stem and root about 1–1½ in. in length, they were ground up in a mortar, 10–20 at a time, and inoculated to French beans. These inoculations were carried out at weekly intervals so that the seedlings used as the source of inoculum were progressively older and larger throughout the experiment. In all, 15 pots of cowpeas and French beans, involving 75 test plants, were inoculated with the macerated seedlings; all the inoculation tests were negative.

In the next experiment the procedure was altered slightly; instead of germinating the seed on blotting paper, it was sown in sterilized sand in the "cellophane" frame previously mentioned. The seedlings were allowed to reach a slightly larger size and to develop 4 leaves before they were inoculated to the test plants. These tests were similarly negative, although occasionally larger seedlings growing under these conditions developed visible symptoms of the disease.

It has been stated that a peculiarity of this virus is its apparent inability to spread beyond the inoculated leaves and to become systemic in the aerial parts of the plant. It was discovered, however, that by injection into the *stem* of the cowpea, the virus could be induced to move about the plant and so to become partially systemic. The presence of the virus, however, in plant tissue seems to be invariably associated with the death of the cells, except in the roots. Such infected cowpeas therefore became moribund and would remain in this condition for months at a time. The living tissues of the stem were killed, but the xylem continued to function, and occasionally the diseased plants remained alive long enough to produce seed; altogether 40 seeds were collected from such plants. Thirty-five were sown and gave rise to virus-free plants, the other 5 together with the pods were ground up and inoculated to test plants. These inoculations were all negative.

The experiment was then repeated, using a different type of plant. It had been found that the bindweed (*Polygonum*) was susceptible to infection with

the virus and also that the virus travelled about in the plant to a certain extent. While not systemic in the usual sense there were foci of infection at intervals in the long stems of this climbing plant. It appeared that a few particles of virus were carried along in the phloem stream until they reached a particular point where they commenced to multiply and produce symptoms of disease (Pl. V, fig. 5). At several such diseased points seeds were formed, in most cases actually on the necrotic stem. This seed was collected and treated in the following ways; part of the ripe seed was sown whilst the green unripe seed together with the rest of the ripe seed was ground up separately and inoculated to test plants. It was found impossible to demonstrate the virus in the *Polygonum* seed by this means; the seed which was sown gave rise to virus-free plants whilst the inoculation tests with the ground-up seed were all negative.

In the next test, a number of tobacco plants were raised from seed which had been stored in the laboratory for periods of not less than 3 years. The tobacco varieties of which the roots were tested were as follows: Virginia, Kentucky White Stem and a variety from Southern Rhodesia. Out of 36 plants of the 3 varieties tested, 25 contained the infective principle in the roots. Storage of the seed for long periods, therefore, has no influence on the subsequent presence of the virus in the roots of plants arising from such seed.

Since the thermal death-point of the virus in expressed sap is known to be 72° C. (Smith & Bald, 1935), it was considered worth while to attempt dry-heat sterilization of tobacco seed at temperatures slightly above this thermal death-point. The experiment was performed as follows. A quantity of ripe dry tobacco seed, var. White Burley, was placed at the bottom of a thin-walled test-tube and a thermometer inserted in such a way that the mercury was completely enveloped by the seed and the bulb was not in contact with the glass. The tube was then immersed in a water-bath, without of course allowing the water level to approach near the mouth of the tube. When the thermometer registered 75° C., the tube and its contents were kept at that temperature for 10 min.

The time required for the germination of seed thus treated was 4 weeks longer than that for the control seed, but the percentage of germination did not appear to be much reduced. The roots of 34 plants arising from the heated seed were tested for virus, and of these 18 contained the virus.

It must be realized, of course, that there is no evidence that the thermal death-point of the virus in expressed sap is the same as the thermal death-point of the virus in the seed. There is, indeed, surprisingly little knowledge of the effect of heat upon viruses within the cells of the host plant. In the next section, therefore, an experiment is described which shows the effect of heat upon the virus in the roots.

V. The effect of heating the virus in the roots of tobacco plants

The roots of a number of normal tobacco plants, var. White Burley, were removed, washed and carefully dried on the outside. A portion of the roots of each plant was then divided into equal parts, one part being macerated and inoculated directly to French beans as a control, while the other part was heated for 10 min. at 75° C. The method of heating was the same as that used for the experiment with heated seed. The piece of root was wrapped closely round the bulb of the thermometer which was inserted in a thin-walled tube. The lower part of the tube was then immersed in a water-bath and kept for 10 min. at a temperature of 75° C. After this the heated root was macerated and inoculated to French beans. The results of this experiment showed that there was much less virus in the heated portion of the roots and in some there was no virus. The following data of the test inoculations illustrate this; plant No. 1, unheated portion of roots 6 lesions, heated portion no reaction; plant No. 4, unheated portion of root 69 lesions, heated portion 2 lesions; plant No. 8, unheated portion 200 lesions, heated portion 4 lesions; all on French beans. The experiment is not exactly comparable to the preceding one with the heated seed because it was not possible to eliminate the moisture in the roots, so that in effect the roots were subjected to moist heat while the seeds were subjected to dry heat. It is not certain, therefore, whether it is permissible to conclude from the results of this experiment that the hypothecated virus in the seed was inactivated by heating to 75° C. Nevertheless, it is interesting to note that exposure of root tissue containing the virus to temperatures of 75° C. inactivated the virus when present in small quantities and reduced it to a trace when present in high concentration.

VI. Tests for the presence of the virus in the stems and leaves of healthy tobacco plants

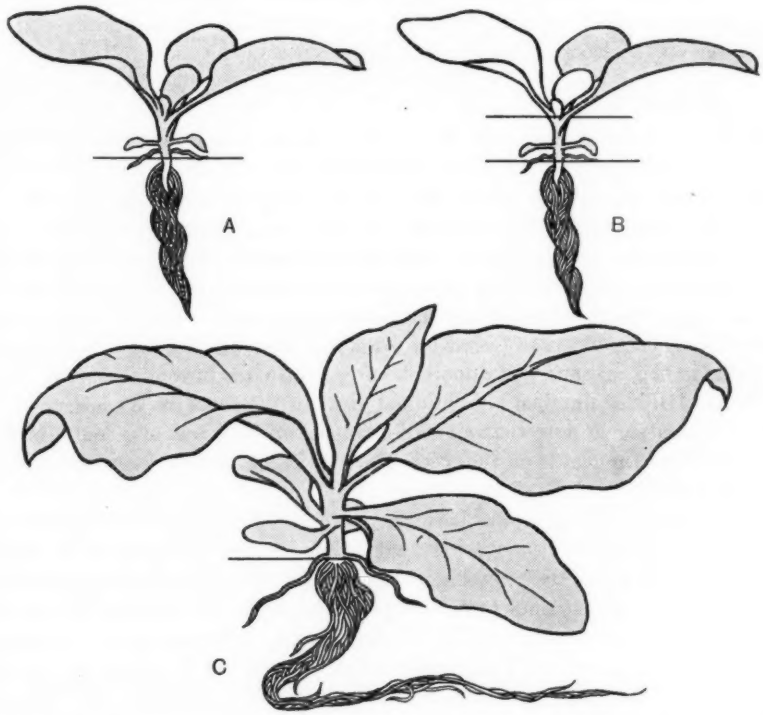
A large number of inoculation tests were made from the leaves and stems of healthy tobacco plants of varying sizes. In the first experiment the tops and roots of 10 large White Burley tobacco plants growing in the field were pounded in muslin bags in a mortar and the expressed juice from each was inoculated separately to cowpeas. All the tests with the tops of these 10 plants were negative, but the roots of 9 of them gave a virus reaction.

In the second test the sap was expressed from the tops of 48 medium-sized White Burley tobacco plants and inoculated to French beans and cowpeas; all these plants gave a negative reaction.

From the large number of negative tests it is clear that the virus is rarely detectable in the stems of symptomless tobacco plants. Nevertheless, on a few occasions the virus has been found in very small quantities in the stem without visible signs of its presence, generally low down near the soil level, and usually in plants growing in the winter.

VII. THE APPEARANCE OF THE VIRUS IN THE ROOTS OF TOBACCO PLANTS PREVIOUSLY VIRUS-FREE

It was discovered in the course of the work that a tobacco plant which had given a negative virus reaction when the roots were tested a first time might contain the virus in the roots in quite large quantities when these were



Text-fig. 1. A. This illustrates the type of tobacco seedling used in the experiments on the appearance of the virus in roots previously virus-free. The horizontal line indicates the point where the roots were severed. B. This drawing indicates where the cuts were made in the experiments in which both top and roots were tested for virus. Severed portions are left unshaded. C. This represents the relative size of the tobacco plant at the time of the second test some 5 or 6 weeks after the first test for virus was made. The horizontal line shows where the roots were severed.

tested a second time some weeks later (Smith, 1935). To investigate the matter further the following experiments were performed. In the first experiment the roots of normal White Burley tobacco plants were carefully washed and cut from the plant with the exception of a single root which was left attached

to the stem in the manner shown in Text-fig. 1 A. The detached roots of each tobacco plant were then ground up in a mortar and the extract inoculated separately to a pot of 5 cowpeas or French beans. By this means 73 tobacco plants were selected which, by inoculation tests, apparently contained no virus in the roots. These plants, each with a single root strand attached, were then re-potted in fresh sterilized soil and allowed to grow on. Some 5 weeks later, when the plants were about 6 in. across and had developed a new root system, the roots were tested for virus again by the same method. Out of the 73 plants which gave a negative virus test the first time, 42 plants gave a positive virus reaction on the second test. The amount of virus in the roots of the different plants can be gauged roughly from the fact that the number of lesions on the test plants varied from 1 to 135.

The next experiment was of a similar nature, but in addition to a first test for virus being made of the roots, a first test was also made of the stem and leaves. A cut was made through the stem in the manner shown in Text-fig. 1 B, as well as the cut through the root as shown in Text-fig. 1 A. Both tops and roots were then ground up and the resulting extracts inoculated separately to test plants. The plants were then repotted in fresh sterilized soil with 1 or 2 leaves and a single root strand attached. After about 6 weeks the stems and roots were tested for virus a second time. Twenty-four plants treated in this manner, and apparently free from virus in root and stem, were selected. Of this number 17 contained virus in the roots on the second test. It is interesting to note that a small quantity of virus was also found in the stems of 2 of the plants on the second test, although on the first trial no virus was detectable in these stems.

The third experiment was performed as follows. The tops of a number of large healthy tobacco plants were cut off and struck as cuttings in baked sand. The object of the experiment was to ascertain whether virus would be present in the adventitious roots formed at the cut surface of the stem. Before the cuttings were plunged in the sand the following tests were made. A section of the stem of the plant, immediately below the place from which the cutting was taken, was tested for virus, and further, in the case of 6 of the cuttings, the roots of the plants from which the cuttings were taken were also tested for virus. The experiment, then, as finally carried out, consisted of 11 cuttings, all of which had been previously tested for the presence of virus in the stem with negative results. In addition the roots of the parent plants of 5 cuttings had given negative virus reactions while the virus was present in the roots of 1 parent plant. The roots of the other 5 of the 11 parent plants were not tested for virus. All the cuttings put out adventitious roots and produced normal plants. Tests of the roots made some 2 months later revealed the presence of virus in 9 of the 11 plants tested.

VIII. PLANTS SUSCEPTIBLE TO INFECTION WITH THE VIRUS BY ARTIFICIAL INOCULATION

No systematic test of the range of plants which can be infected artificially has been made. Those tests which have been made, however, seem to indicate that the range is wide. The following plants have reacted to inoculation with the virus on the leaves.

Cruciferae

Wild radish (*Raphanus* sp.): small "water-clear" necrotic lesions developed on the rubbed leaves, 3 days after inoculation (Pl. V, fig. 4). There was no systemic spread of the virus. A few inoculations to young cabbage leaves gave negative results.

Polygonaceae

The dock (*Rumex obtusifolius* Linn.): numerous small red necrotic lesions appeared on the inoculated leaves. There was no further spread of the virus. Black bindweed (*Polygonum convolvulus* L.): this plant was more susceptible to infection, and, as described on p. 75, some spread of the virus followed inoculation. Local lesions in the form of single or double rings developed on the inoculated leaves (Pl. V, fig. 3).

Chenopodiaceae

Spinach (*Spinacia oleracea*), white goosefoot (*Chenopodium album*): on these two plants only local lesions resulted; the lesions were similar in character on both species and consisted of numerous small white necrotic spots. On sugar-beet, local lesions developed only on the youngest leaves and not on the older leaves. They consisted of small reddish necroses which followed the veins to a certain extent.

Cucurbitaceae

Seedling cucumbers developed numerous necrotic spots on the inoculated leaves.

Grossulariaceae

Red currant (*Ribes*): numerous small "water-clear" lesions developed on the youngest leaves when inoculated, but it was not found possible to produce lesions on the older leaves.

Leguminosae

Cowpea (*Vigna sinensis*) and French bean (*Phaseolus vulgaris*) are extremely sensitive to inoculation with the virus. Dark red lesions develop on the inoculated leaves of the cowpea, 3 days after rubbing on the virus suspension. The virus then spreads through the leaf, producing in the vascular system a dark red necrosis (Smith & Bald, 1935). As a rule the leaf then

drops off and no further infection ensues; occasionally, however, the virus passes up the petiole into the stem. This results in the destruction of the living cells of the stem although the xylem is usually, but not always, left intact. In the latter case portions of the plant remain green and normal while the rest of the plant is moribund. Above 80° F. the production of lesions in the cowpea and subsequent spread of the virus through the leaf are almost inhibited (Pl. IV, fig. 4).

A somewhat similar sequence takes place in the French bean, although this plant is even more sensitive to and intolerant of the virus. Because of the rougher surface these leaves afford a greater number of entry-points for the virus, and the number of lesions produced on rubbing with the virus suspension is very great; if the virus concentration be high they may cover the leaf completely (Pl. IV, figs. 1 and 3). The lesions develop with great regularity 48 hours after inoculation at temperatures of 60° F. Soon after the formation of the lesions, the virus begins to spread through the network of veins on the leaf, producing a rather curious web-like effect (Pl. IV, fig. 2). However small the quantity of virus present, it eventually permeates the entire leaf which becomes completely necrotic, rapidly shrivels and drops off, leaving the rest of the plant healthy (Pl. IV, fig. 3). There is a rather curious phenomenon connected with the spread of the virus in the French bean; when the virus reaches the base of the midrib, it passes a short distance down the petiole till it arrives at a slight natural constriction about 2 cm. from the leaf base. Here as a general rule the virus stops, and the leaf later breaks off at this point. This constriction seems to offer a barrier to the further progress of the virus. Occasionally the virus passes the constriction and enters the stem. When this happens the plant is invariably killed with great rapidity.

The broad bean (*Vicia faba*) is also susceptible but less so than the two foregoing species.

Compositae

Both the common thistle (*Carduus* sp.) and the sow thistle (*Sonchus oleracea*) are susceptible to infection. Small rings and necrotic spots develop on the inoculated leaves, but there is no further spread of the virus.

Primulaceae

It is interesting to find that *Primula obconica* and *Polyanthus* sp. are apparently rather resistant to infection when inoculated on the leaves, although the virus is frequently present in the roots of these two species. *Primula obconica* responded very feebly to inoculation, and after a week the inoculated *Primula* leaves, when ground up and inoculated back to French beans, produced only a single lesion. *Polyanthus* plants gave no visible reaction although twice inoculated with a strong virus suspension, and the tops of the same plants, when ground up and inoculated to French beans a week after inoculation, showed only a trace of virus. The roots of these *Polyanthus* plants,

however, contained the virus in quite large quantities, but it is not possible to say whether this was due to the inoculations or whether the virus had been there all the time.

Solanaceae

Nicotiana tabacum: inoculation was made to a variety of tobacco, Ambalema, which is resistant to infection with the virus of tobacco mosaic; lesions comparable to those formed on other varieties of tobacco developed on the leaves of this variety also. There was no further spread of infection.

Solanum tuberosum: all attempts to infect a number of well-known varieties of potatoes have failed, but inoculation to the young leaves of a seedling potato produced a few dark necrotic lesions. No further spread of the virus took place.

For a description of the symptoms produced on a number of other solanaceous plants, the reader is referred to the previous paper (Smith & Bald, 1935).

Plantaginaceae

Plantain (*Plantago major*): large red lesions developed in 4 days on the inoculated leaves of plantain but infection was confined to these leaves (Pl. V, fig. 4).

In the foregoing brief descriptions of the symptoms caused by this virus on a number of different kinds of plants, it will be noticed that the type of symptom produced in every case is the same. The reaction of the host plant is always in the form of local necrotic lesions whereby the cells are destroyed, the virus never produces any kind of mosaic mottle. Similarly, in none of these host plants is there a true systemic infection which is characteristic of the majority of plant viruses, and in only one or two cases does the virus move out of the inoculated leaf unless indeed it makes its way down to the roots which in most cases are able to harbour the virus without any symptoms of disease.

IX. DISCUSSION

There can be no doubt that this virus is of unusual interest and importance. Moreover, it differs in its behaviour from most other plant viruses in several respects, notably in its restriction to the roots of various plants which are apparently perfectly normal and which usually remain so throughout life; in its inability to become systemic in the host plant when inoculated into the leaves; and in the fact that it will produce symptoms in the leaves of many species of plants if inoculated into them, notwithstanding the fact that it is already present in the roots of the same plant. In other words, it is possible to inoculate a perfectly healthy plant with an extract of its own roots and to produce a severe although localized disease.

The chief interest, however, lies in the questions of the origin of the virus and how it gets into so many different kinds of plants in view of the facts mentioned in the preceding paragraph. It must be admitted that the experi-

ments described in this paper do not answer any of these questions though they make permissible certain speculations.

There seem to be three possible explanations of the infection, and these will be discussed in the light of the foregoing experiments. These explanations are as follows. First, it may be assumed that the virus is present all the time in the stem, but present either in a non-virulent form which requires to gain virulence by possible alteration in particular cells of the roots or else in a dilution too great to give a positive reaction on inoculation. This theory, of course, also involves seed transmission of the virus in undetectable form or quantity, since the presence of the virus cannot be detected in the seed by available inoculation methods. The second possible explanation is the existence of a mode of virus transmission unknown to plant virus workers, while the third possibility is that the infective principle is arising spontaneously within the plant or is being manufactured by it.

The theory of seed transmission of the virus would, of course, explain a good deal which is at present obscure, but what evidence there is seems against this theory. The facts that the infective agent cannot be demonstrated in the seed, seed capsules or flower petals of tobacco plants and in most cases, as in tomatoes, *Polyanthus*, etc., never seems to leave the root at all, are strongly against the possibility of seed transmission. In the 9 years during which tomatoes have been cultivated in the insect-proof glasshouses at Cambridge, not one tomato plant has been observed to show *naturally* the symptoms which are typical of this virus in the leaves, yet the virus appears to be present in the roots of a fairly high percentage of normal tomato plants. Again, if the virus is present in the seed in undetectable form, it is reasonable to suppose that it would multiply sufficiently in the roots of seedlings germinated on blotting paper to give a positive reaction, but this has never been demonstrated.

The second possibility, i.e. that some other means of spread of the agent exists, is perhaps the most likely explanation of the problem. If, however, the soil is concerned in the dispersal of the virus, then there must be some additional means of dissemination, either by air or water, which enables the infective agent to reach the soil after this has been sterilized. It should be remembered that the virus occurs not only in the roots of plants growing in unsterilized soil but also in the roots of plants, previously shown to be virus-free, growing in autoclaved soil in pots which have been boiled.

It seems extremely unlikely that the virus is insect-borne, for the following reasons. The incidence of the disease is highest at a time of year when insects are absent. Fairly extensive tests with all the normal insect and allied fauna of glasshouses have given negative results, and, indeed, the very nature of the disease itself makes it extremely difficult for an insect to transmit it, since a potential vector must feed either upon the roots or on the necrotic lesions themselves in order to pick up the infective agent. It is a significant point that young tobacco seedlings growing on blotting paper *inside* Petri

dishes have never given a virus reaction while larger seedlings growing in sterilized sand or soil in the cellophane frame and subjected to the ordinary routine have frequently given a positive virus reaction and also shown symptoms of disease. Seedlings which show symptoms of infection contain the virus in the roots in very high concentration, and this, of course, may be the reason why the virus is forced up into the aerial parts of the seedling. However, the question of external contamination cannot be definitely settled until the experiments now in progress on the cultivation of tobacco plants in sterile media are completed.

The third possible explanation is the theory that the infective agent is already in the roots and is therefore arising in the plant or is manufactured by it. This is a convenient theory and one that also fits the facts. After all, is such a suggestion so unlikely as it may appear at first sight? Is it not possible that certain proteins normally present in the roots may, under stimulation, lend themselves to a slight rearrangement of the molecules so that they become pathogenic or autocatalytic? In other words, an agent is thereby produced which, as a previous theory has it, compels the cell to produce more of this agent or, alternatively, finds in the cell more protein so similar in nature as to be easily converted into more of itself. This theory may seem fantastic if the virus be considered as a "living organism", but what evidence is there that this particular infective principle is "living" in the accepted sense of that word? The fact that it is present in the roots of such a large number of apparently normal and totally dissimilar plants, chosen entirely at random, seems in the writer's opinion strongly against the possibility of seed transmission and the other modes of dissemination common to most plant viruses. This agent, then, may possibly be, not perhaps a normal constituent of plant roots, but an entity easily produced under certain abnormal conditions, and here it may be stated that the disease seems in some way connected with the glasshouse environment. Again, it must be remembered that with slight exceptions this agent is entirely harmless to the plants unless removed from the roots and introduced into different surroundings.

These speculations, however, must wait upon fresh discoveries to show which, if any, of these theories is the correct one.

X. SUMMARY

An account is given of a virus which is present in the roots of a number of different plants. Plants which have been shown, so far, to contain the infective principle in the roots are several members of the Solanaceae, tomatoes, tobacco, etc.; of the Primulaceae, *Polyanthus* sp. and *Primula obconica*; of the Geraniaceae, zonal Pelargonium; and of the Compositae, the aster. Such plants show no indication of infection with the exception of *Nicotiana tabacum* and *N. glutinosa*, which may develop natural disease symptoms. These symptoms are usually confined to the two lowermost

leaves and develop most frequently under conditions of low temperature and low light intensity. The virus never becomes completely systemic in any host plant and is generally restricted to the inoculated leaves. Experiments are described which show the virus to be present in the roots of tobacco plants which had given a negative virus reaction a few weeks earlier. The virus is found in the roots of tobacco plants arising from seed which has been heated to a temperature of 75° C., the thermal death-point of the virus in expressed sap being 72° C. All attempts to demonstrate the presence of the virus in the seed have proved negative.

Although no systematic study has been made, the range of plants susceptible to local infection in the leaves, by artificial inoculation, seems wide. Tests have shown that about 25 species of plants belonging to 10 families respond to inoculation with the virus.

Three possible explanations of the origin and spread of the infection are discussed.

Acknowledgments

Acknowledgment is due to the writer's friends and colleagues at the virus research station and elsewhere, with whom many points in this work have been discussed; valuable suggestions have also been made by Mr F. T. Brooks, F.R.S., Dr E. J. Butler, F.R.S. and Prof. D. Keilin, F.R.S., to whom the writer expresses his best thanks.

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EXPLANATION OF PLATES

PLATE IV

Fig. 1. Leaf of French bean (*Phaseolus vulgaris*) inoculated with an extract from the roots of a normal-looking tobacco plant. Symptoms develop within 48 hours at temperatures of about 60° C. The leaf shown in the illustration was inoculated 72 hours before being photographed.

Fig. 2. Leaf of French bean, showing the gradual spread of the virus which etches out the vascular system as it moves. In the leaf illustrated, there was a single lesion which can be seen at the top, slightly to the left of the mid-rib. From this single lesion the virus has gradually permeated the area shown in the photograph.

Fig. 3. Two plants of French bean inoculated on the first leaves with the virus; note that the uninoculated portions of the plants are free of virus.

Fig. 4. Photographs showing the effect of heat upon the development of the virus in the plant; both leaves were of the same age and were inoculated at the same time with the same inoculum. The upper leaf was from a plant which had been kept at a temperature not exceeding 65° F., the lower leaf from a plant kept at temperatures between 85–90° F. In the latter case not only was the number of lesions much smaller but the spread of the virus in the leaf was inhibited.

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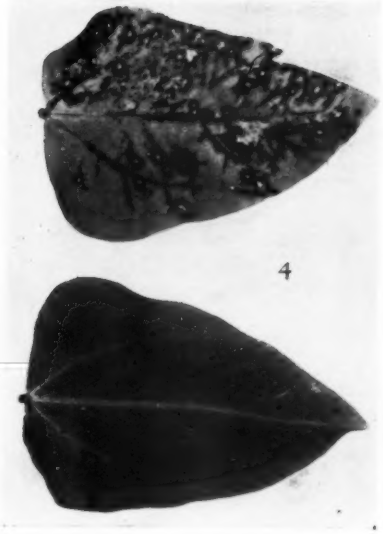

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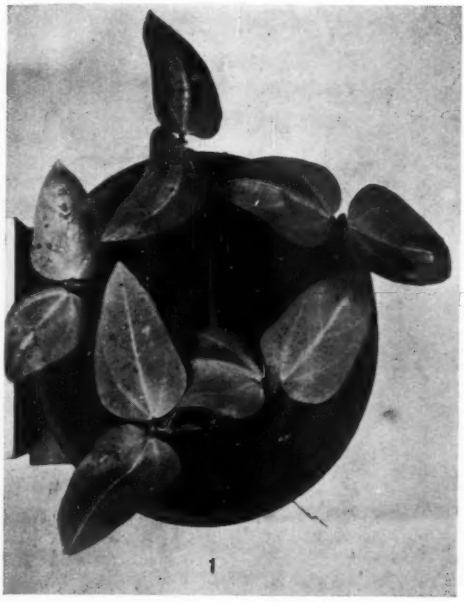
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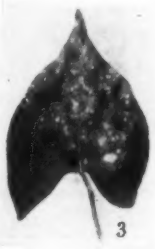
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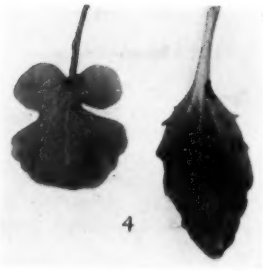
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PLATE V

Fig. 1. Cowpeas (*Vigna sinensis*) inoculated from the top and roots respectively of a healthy White Burley tobacco plant; the three plants on the left were inoculated with an extract of the roots and show numerous lesions, the two plants on the right were inoculated with an extract of the leaves and stem and are quite healthy.

Fig. 2. Leaf of White Burley tobacco plant inoculated with the virus and showing the type of lesion sometimes produced.

Fig. 3. Type of local lesion produced on the leaves of bindweed (*Polygonum*).

Fig. 4. Local lesions produced, left on wild radish (*Raphanus* sp.) and right on plantain (*Plantago major*).

Fig. 5. A single systemic lesion on a leaf of *Polygonum*; this leaf was separated by about 12 in. of stem from the inoculation point. The lesion was probably formed by a few particles of virus which had been carried along in the phloem stream and had multiplied at that point. The lesion is visible at the extreme right-hand edge, towards the tip of the leaf.

(Photographs by J. P. Doncaster)

(*MS. received for publication* 18. I. 1936.—Ed.)

FURTHER STUDIES ON A VIRUS FOUND IN THE ROOTS OF CERTAIN NORMAL-LOOKING PLANTS

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(With Plate VI)

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I. Introductory

In previous communications (Smith & Bald, 1935; Smith, 1935, 1936) a new and unusual plant virus has been described. This virus was found to be present in the roots of a number of normal-looking tobacco plants, shown to have been originally virus-free, under conditions which, up to the present, have been considered to be proof against virus infection. Three possible explanations of the presence of the virus in the roots were put forward. These theories are as follows:

(1) The virus was being manufactured by the plant itself.

(2) The virus was seed-borne in undetectable form or quantity and needed the particular environment of the root for its multiplication or conversion into demonstrable condition.

(3) The virus was reaching the plants by a method of dispersal or dissemination hitherto unknown in plant-virus study.

The present paper describes experiments designed to test each of these three theories. The French bean (*Phaseolus vulgaris*) was used throughout as a test plant for the presence of the virus.

In the description of the experiments, the phrase "sterilized soil" refers to soil heated in an electric sterilizer to a temperature of 100° C., wet heat, for 2 hours. This sterilizer was shown experimentally to destroy the virus in the soil. Soil heated to 120° C. for 20 min. is referred to as "autoclaved soil".

II. EXPERIMENTS TO INVESTIGATE THE QUESTION WHETHER THE VIRUS CAN ARISE *DE NOVO* WITHIN THE PLANT

It was considered that if susceptible plants were grown from seed under bacteriologically sterile conditions, this would at least preclude the possibility of external contamination, and if such plants were still found to contain the virus, then the possible explanations of infection would be reduced to those of seed transmission or heterogenesis.

The experiments were of two kinds and were planned as follows. For both types of experiment the tobacco plant var. White Burley was employed. Under the conditions obtaining in the writer's glasshouses, 80–100 per cent of the normal healthy stocks of this plant contain the virus in the roots.

In the first type of experiment the seed was sterilized externally by several washings in a 0·25 per cent solution of corrosive sublimate, followed by several washings in sterile water. The seeds were then transferred under aseptic conditions to a sterile nutrient agar. Some difficulty was experienced in finding a nutrient agar in which the tobacco plant would grow satisfactorily, the one finally chosen being made up as follows:

Calcium nitrate	1·0 g.
Potassium acid phosphate	0·25 g.
Potassium nitrate	0·25 g.
Magnesium sulphate	0·25 g.
Ferric chloride	A trace
Agar	20·0 g.
Distilled water	1000 c.c.

The plants were grown in boiling tubes and 2000 c.c. Erlenmeyer flasks which were placed in the glasshouse. The plants were allowed to grow for a minimum period of 3 months before the roots were tested. Altogether the roots of thirty-five plants grown in this manner were tested for virus, all these gave a negative virus reaction. It would be quite impossible to test this number of tobacco plants, grown in the normal way in the glasshouses at Cambridge, without finding the virus present in the roots of at least 30 per cent.

The second type of experiment involved the use of tissue-culture methods, and the technique of White (1934) was closely followed. A root was detached from a healthy tobacco seedling and divided into two unequal portions. The larger piece was ground up and inoculated to French beans as a test for the presence of the virus, while the smaller piece, between 2 and 3 cm. in length and bearing several small side roots, was set aside for culturing. If the test for virus was negative, the second piece of root was washed in two or three

changes of a 0·25 per cent solution of corrosive sublimate then rinsed in sterile water and transferred under aseptic conditions to the sterile culture medium recommended by White (1934).

In growing the pieces of root in the culture medium it was found absolutely necessary to sterilize them externally in the corrosive sublimate. If the roots were washed with sterile water only, contaminations of some kind invariably resulted. The weak solution (0·25 per cent) of corrosive sublimate used, however, sterilized the roots perfectly without injury and there was no subsequent contamination. The pieces of root grew well under these conditions, and after 3–4 months were removed from the culture medium and tested for virus. Altogether 20 tests were made in this manner and in every case a negative result was obtained.

These two experiments prove, therefore, that the virus is not arising *de novo* in any part of the plant, and moreover they show that tobacco plants, when protected from any external influences, do not contain the virus. The first experiment also offers further proof that the virus is not seed-borne.

III. Experiments on the question of virus transmission through the seed

The numerous tests which have been made already (Smith, 1936) indicate that the virus is unlikely to be seed transmitted. It may be of interest, however, to describe an experiment in which the seeds of two species of susceptible plants were inoculated with the virus and then sown. Seeds of the French bean (*Phaseolus vulgaris*), after being soaked in water for 24 hours, were inoculated with a sap suspension of the virus. They were then potted up in sterilized soil. In three plants of the twelve arising from the seeds thus treated, a necrotic lesion appeared above the soil level and gradually made its way up the stem (Pl. VI, fig. 5). The stem then rapidly became almost completely necrotic with a deep longitudinal fissure along its whole length. These three plants soon died. The other nine plants remained normal in appearance although the virus was present in the secondary roots of four of them. This infection, however, had no connexion with the inoculations, since the control plants showed an equal contamination with the virus. Evidently in the case of the three plants which showed symptoms the virus had been introduced directly into the vascular system. The same type of symptom can be produced by inoculating the virus into the stems or tap roots of young bean plants.

A number of tomato seeds were then treated in a similar manner, but the inoculation process had a deleterious effect on the germination and only seven plants were obtained. These seven plants were quite normal in appearance and five contained the virus in the roots. Here again, however, no deduction can be drawn from this fact, since the control plants showed an equal amount of infection.

This experiment seems to suggest, so far as the bean seeds are concerned,

that if the virus were present in the seed the resulting plant would show symptoms of disease, and *natural* symptoms of disease in French beans caused by this virus have never been observed.

IV. EXPERIMENTS TO SHOW THAT THE VIRUS DOES ARRIVE IN THE PLANT FROM EXTERNAL SOURCES

In the first experiment in this series, seed of White Burley tobacco was sown in two large covered crystallizing dishes, one containing sterilized soil and the other unsterilized soil. In both cases the soil was first moistened with tap water and the dishes were not again opened. In the controls the same seed and soil were used, but the dishes were left open and subjected to the ordinary glasshouse routine.

The virus was first detected in the roots of the seedlings growing in the open dish containing sterilized soil on 31 March, i.e. 27 days after the seed was sown. The seedlings in the unsterilized soil in the open dishes were first found to contain the virus on 7 April, i.e. 34 days after the date of sowing. No virus could be detected in the plants growing in the covered crystallizing dishes. This experiment was repeated with the same result.

In the next experiment seed of White Burley tobacco was sown in various types of soil obtained from different localities. The soils were put into sterilized boxes and the plants grown in the glasshouse in the usual manner. The boxes were left uncovered and contained the following soils, etc.: (*a*) sterilized soil, (*b*) unsterilized new soil, (*c*) soil from the writer's garden, (*d*) soil from a field on the University farm, (*e*) silver sand which had been baked over a Primus stove. The silver sand was watered with an autoclaved nutrient solution.

The virus was found in the roots of the tobacco plants growing in all four kinds of soil and in the silver sand. The percentage of infection, about 70 per cent, was highest in the plants grown in the sterilized soil and lowest in the plants grown in the silver sand.

The experiment was then repeated under slightly different conditions. In this case the tobacco seed was exposed for 15 min. to a temperature of 75° C. dry heat. This treatment delayed the germination of the seed by about 3 weeks but did not appreciably affect the percentage of germination. After being heated the seed was sown in baked silver sand contained in a sterilized box and the resulting seedlings were later transplanted to (*a*) sterilized soil, (*b*) autoclaved soil, (*c*) autoclaved soil to which had been added a cell-free filtrate of the virus, (*d*) baked sand. These different soils were contained in sterilized pots and the plants were grown in the glasshouse in the usual manner. The results of this experiment were as follows. All the plants tested from the sterilized soil and the autoclaved soil, to which virus had been added, contained the virus in the roots. Of the plants repotted in autoclaved soil three out of twelve were infected, and of the plants in baked silver sand five out of fourteen were infected.

It is not claimed that heating the tobacco seed to 75° C. dry heat would destroy any virus which might be inside the seed. It is probably not possible to do this without also destroying the germinating power of the seed.

Another experiment on these lines was then carried out in which eight small White Burley tobacco plants which had been proved by inoculation tests to be virus-free were repotted in autoclaved soil contained in boiled pots. These were allowed to grow for about 5 weeks and were then tested again; of the eight plants three gave a positive virus reaction on the second test.

The results of the foregoing experiments prove that the virus is a contamination which reaches the roots of the plants from some external source.

V. Experiments to decide whether infection takes place through the soil or other medium in which the plants are growing

The experiments described in this section were designed to investigate a possible relationship between infection and the soil or other medium in which the plants are growing at the time of infection.

Pieces of stem of normal-looking White Burley tobacco plants were cut into three equal pieces and the portion lowest on the stem was ground up and inoculated to the test plants. If no virus was present in the stem, one of the two remaining pieces of stem was rooted in sterilized soil and the other piece was rooted in water culture. Owing to the difficulty of finding a liquid medium in which the cuttings would root properly, they were grown in test-tubes containing water from the tanks. The cuttings grew remarkably well under these conditions and formed quite an extensive root system. Some weeks after the cuttings had become well established in soil and water respectively, the roots were tested for virus. Of nine cuttings rooted in soil five contained the virus in the roots, none of nine cuttings rooted in water was infected (Pl. VI, figs. 1 and 2). This experiment seems to suggest that soil or sand is a necessary, though possibly only mechanical, agent in the process of infection. This is further borne out by the facts that French beans rooted on moist blotting paper and grown in open crystallizing dishes have never been found to contain the virus in the roots, even if, as in one case, the blotting paper was moistened with a virus suspension. The virus also seems unable to enter the roots of cuttings growing in water culture. It appears therefore that some wounding must be necessary for infection, and this condition might be fulfilled by the breaking of root hairs as the root makes its way through the soil or sand.

The next experiment was to test the longevity of the virus in the soil when this agent was added experimentally in a cell-free filtrate. The first test was made as follows. A sterile 5-in. pot was filled with sterilized soil and 10 c.c. of virus filtrate were added. The pot was then placed on a glass plate covered with a bell-jar and kept in the glasshouse. Inoculations were made at intervals from the soil in the pot. In the first test positive virus reactions

were obtained for 5 weeks, although the number of local lesions on French bean leaves continued to show a steady fall. In the second test which was carried out in a similar manner, twenty-six inoculations were made, covering a period of 48 days. These inoculations exhausted all the soil which remained infective till the end of the experiment. A third test was carried out in which baked sand was substituted for the sterile soil, in this case the virus remained infective for 7 weeks, so far as tested.

Finally the experiment was repeated under bacteriologically sterile conditions. A number of test-tubes containing soil were autoclaved and about 5 c.c. of a bacteriologically sterile virus filtrate were added to each tube. These were stored at laboratory temperature, and the contents of one tube were inoculated to the test plants at weekly intervals. The absence of bacteria or any living tissue in the soil seemed to make no difference to the longevity of the virus which remained infective under these conditions for 11 weeks, so far as tested.

Similar tests with two other viruses were carried out for comparison. The viruses used were potato virus X and tobacco virus I, and the conditions of the experiments were the same as for the first virus. Potato virus X remained infective in the soil for 24 hours only, while tobacco virus I could still be detected in small quantities in the soil up to 24 days.

These experiments show not only that the virus is capable of remaining infective in soil for long periods in the absence of any living tissue, but also that it is easy to demonstrate its presence in the soil after it has been added experimentally.

There are, however, one or two anomalies in regard to the presence of the virus in the soil which are interesting. It is exceedingly difficult to find the virus in the ordinary supplies of potting soil before the tobacco or other plants are growing in it. When the tobacco plants are in the soil, however, it is quite easy to demonstrate the presence of large quantities of virus. Here, of course, the chief difficulty is to be sure, in making inoculations from the soil, containing infected plants, that no fragment of infected root tissue is also present in the soil inoculum. The following experiment illustrates this phenomenon. A quantity of fresh soil was twice sterilized and put into two sterilized seed boxes. One box was sown with White Burley tobacco seed, the other box being left unsown, both boxes were placed side by side in the glasshouse and subjected equally to the ordinary routine treatment as regards watering. About 4 weeks after the germination of the seed, virus was found in the roots of the tobacco seedlings and further tests continued to show a high percentage of infections. Out of seventeen tests made at intervals of a few days eleven gave a positive virus reaction. Out of seventeen precisely similar tests made from the box containing soil only, a single virus lesion developed on the test plants at the fifteenth attempt, nearly 3 months after the experiment had been started. Presumably if the virus is arriving in the soil from an external source it should occur equally in both boxes, and it is rather curious that its

presence can only be demonstrated in the soil in the absence of plants with such great difficulty. This difficulty is also shown by the large number of negative inoculations which have been made. Out of more than 100 trials with the soil only three lesions in all have been observed on the test plants, and these developed from inoculations made with sterilized soil which had been lying untouched in the bin but exposed to the atmosphere for 9 weeks.

VI. Experiments to test whether the virus is water-borne

The data so far obtained from the foregoing experiments seem to justify the assumption that the virus is either water- or air-borne or both, and that having reached the soil by this means it sooner or later invariably enters the roots of plants growing in the soil.

In the first experiment in the series to test if the virus is water-borne, fourteen seeds of the French bean were planted in pots containing sterilized soil previously moistened with a suspension of the virus in water. The control consisted of fourteen seeds planted under similar circumstances but without the addition of the virus to the soil. The results of this experiment were as follows. Of the fourteen French beans growing in the soil moistened with virus, twelve gave a positive reaction, and of the fourteen control plants, eleven contained the virus in the roots. Since the amount of natural infection is so great as to nullify the results of this experiment, it was repeated with certain alterations. Seeds of White Burley tobacco were sown in two sterilized boxes containing autoclaved soil. One box was watered with boiled tap water, the other was watered from the greenhouse tanks. In this experiment, ten of of the tobacco plants watered from the tanks contained the virus in the roots, but the roots of the plants watered with boiled tap water were virus-free.

The next question to be considered was whether the virus could remain infective in the water tanks. As stated in a previous communication (Smith, 1935) inoculation tests with the water from the tanks had proved negative. This time, however, the sludge at the bottom of the tanks was carefully filtered off and inoculations made from this. The virus was found to be present in small quantities in the sludge where it appears to remain infective for long periods.

These results seem to justify the assumption that the virus is water-borne to the soil whence it makes its way to the roots of the plants. In view, however, of the immense amount of natural infection which takes place and the comparatively small amount of virus detectable in the tanks, it cannot be said definitely that this is the only means of dissemination of the virus, and the question of air-borne infection to the soil must still be considered.

VII. EXPERIMENTS TO TEST WHETHER THE VIRUS IS AIR-BORNE

In these experiments an apparatus was devised, using two large aspirators, by which a more or less continuous stream of air was sucked through 5 c.c. of sterile water. The apparatus was constructed by Mr J. P. Doncaster, to whom the writer's best thanks are due.

About twenty tests, each of 2 or 3 days' duration, were made, but all these have proved negative. The apparatus was placed in the glasshouse close to the experimental plants, so that if any virus was present in the air it should presumably be drawn into the water trap.

So far as they go, then, these tests suggest that the virus is not carried in the air. There is not, however, any apparent reason why it should not be air-borne, when the small size of the virus and its complete resistance to desiccation are considered.

VIII. SOME CHARACTERISTICS OF THE VIRUS

Further studies on the properties of the virus show it to be of a most resistant nature, and it is certainly the most resistant plant virus so far described. It was at first thought that the virus would not stand drying (Smith, 1935), but subsequent work has shown that it may be completely desiccated over sulphuric acid and still retain undiminished infective power for long periods. In the desiccation experiments the virus was precipitated from crude sap or a Kieselguhr filtrate with absolute alcohol, the precipitate was spun down, filtered off and dried in a desiccator over sulphuric acid. It could then be stored at laboratory temperatures and remained infective for 6 months, so far as tested.

The virus is apparently unaffected by alcohol, and it can be kept, if not indefinitely, for very long periods in absolute alcohol. It is still strongly infective after being stored for 6 months in absolute alcohol at laboratory temperature.

Its thermal death-point in extracted sap is low, about 72° C., but it can be heated for 15 min. at 100° C. dry heat without being completely inactivated. Experiment has shown that the virus of tobacco mosaic (tobacco virus I) will also withstand a similar degree of dry heat.

IX. DISCUSSION

This virus is unusual in its behaviour in several respects, notably in its extreme resistance, its mode of dissemination and its ability to remain infective in the roots of a great variety of different plants, some most susceptible to artificial inoculation with the virus, without producing visible signs of its presence. It may be suggested that the virus is a transitional stage between a pathogen and a non-pathogen. With a single exception, i.e. *Nicotiana*, no

natural disease symptoms due to this virus have ever been observed, yet, under the writer's conditions, it is present in the roots of a very high percentage of the normal stocks of such plants as tomatoes, French beans (*Phaseolus vulgaris*), Pelargoniums, *Polyanthus*, etc.

In its method of spread the virus so far seems unique, and it is hoped that its further study may also throw light on the means of dissemination of other viruses of which the agents of spread are unknown. It should perhaps be emphasized that the mode of dissemination of this virus cannot be compared to isolated cases of accidental contamination. A glance at the number of natural infections recorded in the foregoing experiments will show this. On the contrary, the method of spread of this virus seems at least as successful as that of many fungi and bacteria pathogenic to plants. It is interesting that the virus seems confined to glasshouses, at all events it has not yet been found in the roots of susceptible plants growing out-of-doors *unless* those plants had been raised as seedlings in the glasshouse. No systematic test of susceptible plants growing in the open has been made but it is hoped that this may be done in the near future.

There is some evidence that the virus is changing its pathogenic reactions so far as the very susceptible French bean (*Phaseolus vulgaris*) is concerned. For more than 18 months this species has been used as a test plant and invariably the virus was confined to the inoculated leaf. Recently, however, systemic symptoms have developed on the young uninoculated leaves of the test plants (Pl. VI, fig. 3), and in the case of at least one strain of the virus systemic symptoms now frequently develop in inoculated French beans with the result that the plants are killed. The effect of systemic invasion of the plant is the production of "pockets" of virus on the young leaves, and here multiplication takes place. Necrosis spreads out from these foci as it does from the local lesions formed on inoculated leaves. The symptoms of systemic infection are still entirely necrotic; mottling of any sort has never been observed.

X. Summary

The results are described of further studies on a virus found in the roots of certain normal-looking plants. It is shown that the virus is a contamination arriving from outside sources. The virus has been detected in the sludge at the bottom of the water tanks, and the experimental evidence shows that it is water-borne to the soil whence it reaches the roots of the plants. Experiments to test the possibility that the virus is also air-borne have so far proved negative.

The virus is exceedingly resistant, it can be completely desiccated and remains infective in the dry state for many weeks. It is also unaffected by alcohol and is still infective after being stored in absolute alcohol for 6 months.

The virus is thought to be a transitional stage between a pathogen and a non-pathogen.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

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EXPLANATION OF PLATE VI

Fig. 1. French beans inoculated from the roots of one-half of a piece of tobacco stem, originally virus-free, rooted in sterilized soil. Note the numerous lesions. (Compare Fig. 2).

Fig. 2. French beans inoculated from the roots of the other half of the same piece of stem, rooted in water culture. Note that the French beans are quite healthy. (Compare Fig. 1).

Fig. 3. Non-inoculated leaves of French bean infected with the virus. This is one of a number of systemic infections which only commenced to appear after the virus had been cultured in French beans for a year and a half.

Fig. 4. French bean plant inoculated in the stem, showing the vascular system picked out in two parallel lines by the virus. Note that the virus has travelled up the petiole to the left.

Fig. 5. French bean plant arising from a seed which has been inoculated with the virus. Note the longitudinal lesion at the base of the stem where the virus is making its way up.

(Photographs by J. P. Doncaster)

SHEEP MYIASIS IN SOUTH-WEST SCOTLAND, WITH SPECIAL REFERENCE TO THE SPECIES INVOLVED

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(With Three Figures in the Text)

Introduction

The increasing seriousness of sheep myiasis in Scotland, Australia and South Africa has, of recent years, claimed the attention of many workers. During the past 4 years the sudden and alarming aggravation of this pest in Scotland has attracted the notice not only of scientists and of the agricultural world, but of the lay public in general—a state of affairs clearly indicated by the extensive correspondence on this subject conducted by the *Glasgow Herald* during 1934–5. A significant feature has been the appearance of sheep maggot in areas hitherto untouched, while the position of sheep farming in the more seriously affected districts has become increasingly precarious. While, in most farms, the number of fatalities is still relatively small, extreme cases are noted from time to time, as, for example, that of an Argyllshire farm which lost 81 sheep out of 1000 in one season, despite the attentions of three shepherds. In the same year (1934) another farm in the same district lost 26 sheep out of 500. Of even more widespread importance is the cumulative damage to fleeces and to the sheep themselves, with resulting loss of condition and market value. The work of shepherds, moreover, is becoming excessively heavy, necessitating careful examination of the flock for maggot at least once daily during the fly season. Even such precautionary measures have, in some districts, proved entirely ineffective in preventing the occurrence of advanced cases, especially where the sheep are scattered over a large area of rough and bracken-covered country.

While in Australia and South Africa the volume of literature on sheep myiasis has been steadily accumulating, the number of independent investigations in Britain is still comparatively small. This is especially true of Scotland, the most seriously affected part of the British Isles. Since the classic survey by MacDougall in 1909, little of importance transpired until the beginning, in 1932, of the intensive researches of Prof. Ritchie and his colleagues at Aberdeen. It has been established by the above workers that *Lucilia sericata* Mg. is the almost exclusive cause of this condition in Scotland, some even asserting that it is definitely the only fly concerned. There are, however, occasional records of the presence of other species, notably *Calliphora erythrocephala* Mg. and *Lucilia caesar* (L.). It is one of the main objects of the present

paper to emphasize the fact that the larvae of a few common carrion-flies do occasionally occur along with *L. sericata* as sheep maggots, at least in the area under consideration. While the part played by these is still of minor importance, there is every indication that they are following the general tendency of the Calliphorinae to forsake their former relatively harmless mode of life in favour of parasitism. In view of the implied danger, we have considered it important to attempt to estimate the incidence and role of these flies in certain districts, with the object of attracting the notice of authorities in a position to carry out a more widespread survey. As some of the results are not in agreement with the views of such a recognized authority as Prof. Ritchie, it may be noted that, throughout the entire work in 1933–5, the most stringent precautions have been employed to obviate any possibility of contamination of cultures.

While engaged in field observations on carrion-flies in 1933, one of the writers was impressed by their abundance in certain areas where sheep maggots were prevalent, and decided to find, by rearing experiments, whether they played any part in sheep myiasis. The results indicating that such was the case, it was decided to conduct a more extensive investigation, including parts of Ayrshire, Argyllshire and Arran. It was considered better to make a careful examination of the conditions obtaining on a small number of representative farms than to treat a large number more superficially. The farms included (Fig. 3) are: Craigends, Tayburn, Blackbyre, Low Rushaw, Muirside, Cairnhill and Drumtee, Cunninghame, Ayrshire; Glencloy, Glenkill and Machrie, Arran; Beachmeanach, High Crubasdale, and Barr Mains, West Kintyre; Carsaig and Arinafadbeg, Knapdale, and Fearnoch, Cowal. Material has been received from the following farms and districts outwith the area: Kirkton of Crawford, Lanarkshire; Auchmull and Gleneffock, Angus, and Strath, Flowerdale and Kerrysdale, Wester Ross. Fuller accounts of these farms are given below.

METHODS

While most previous work has been done on material sent by farmers for examination, it has been found more satisfactory to collect the larvae personally. This method, though necessitating more expenditure of time and labour, not only furnishes more complete information, but also ensures the purity of the sample, and that the larvae are taken from living sheep—a need not fully realized by many farmers. Practically every collection listed below was so made. In few cases were egg-masses collected, both on account of their fragility, and of the very unwieldy numbers of adults obtained by rearing even a few batches. In view of the high mortality frequently occurring in crowded cultures, it was found preferable, in the case of heavy infections, to rear representative numbers of each instar present than to take a large proportion of the entire number. The obvious drawback to this method is that larvae of a given species, present in small numbers in a heavy infection of *Lucilia sericata*, may be missed entirely, but where it is impossible to rear all the larvae present, the collection of a few of each stage gives a better chance of obtaining representatives of each original batch of eggs. The material was transferred directly

from the sheep to the breeding jars, which were covered by four or five layers of close cotton, linen or muslin, secured by tight bands. The feeding materials employed were various. Minced beef was found to be rather unsatisfactory, owing to its property of forming a solid, comparatively dry cake. Fish, liver and brain, whole or mashed, or a mixture of the three, were found to be excellent. Equally good were the viscera of birds freshly shot for the purpose. As a substratum for pupation, fine sawdust, moss litter and bulb fibre were found effective. Earth and sand usually failed to give good results, as they were found to form dense masses round the puparia, with resultant high mortality. In every case it was found best to keep the material fairly moist—otherwise the resting larvae tended to dry up. In the many cases where larvae collected during late summer or autumn showed a tendency to hibernate, metamorphosis was accelerated by exposure to higher temperatures. It is both inconvenient and unsatisfactory to allow specimens to hibernate under artificial conditions, as no matter what precautions are taken, a large number usually die during the winter.

Though the apparatus devised by Keilin (1924) is beyond doubt the most satisfactory, as most of the work was done actually on the areas under investigation, and at considerable distance from any laboratory, it was necessary to use cages not only flyproof but portable. Various designs and materials were tried, the most efficient and generally used being the following: a large case, $26\frac{1}{2} \times 18\frac{1}{2} \times 13$ in. externally, was built of $\frac{3}{4}$-in. dove-tailed white mahogany, which does not warp readily, with a carrying strap passing over the sides and top (Fig. 1 A, B). The door, of nine-ply wood, formed one of the large sides, and was hinged below. The edge of the door was bevelled to fit closely when shut, and the fitting was further secured by a small, inwardly projecting rim within the door, ensuring an absolutely tight joint (Fig. 2). The whole was closed by means of three jam-hasps, which kept the door pressed tightly shut and flush with the surface. The case was adequately ventilated by means of nine slots $3\frac{1}{2} \times \frac{3}{4}$ in., six in the door, and three in the opposite side. These were covered by wire gauze of the finest grade, reinforced by a protective beading round the edge. Within were three sliding shelves of nine-ply wood, which could be entirely withdrawn, and, being provided with a deep flange at each end, could stand separately without disturbing the jars. Each shelf was perforated by seventeen staggered round holes. Glass tumblers, on account of their lightness and of their slight taper, which helped them to sit securely in these apertures, were used throughout as breeding jars. The tumblers and their covers were nowhere in contact with each other or with the walls of the case. This being a new type of apparatus, its flyproof qualities were tested thoroughly in the following way before it was used for rearing sheep maggots: twenty-one jars containing pure separate cultures of the larvae of six different species of carrion-fly were placed side by side in the box, along with a large number of open jars containing uncovered beef. The case was then closed, and left in a sheltered, sunny place, much frequented by blow-flies, for the space of 1 week. Large numbers of flies were attracted by the smell, but at the end of the week

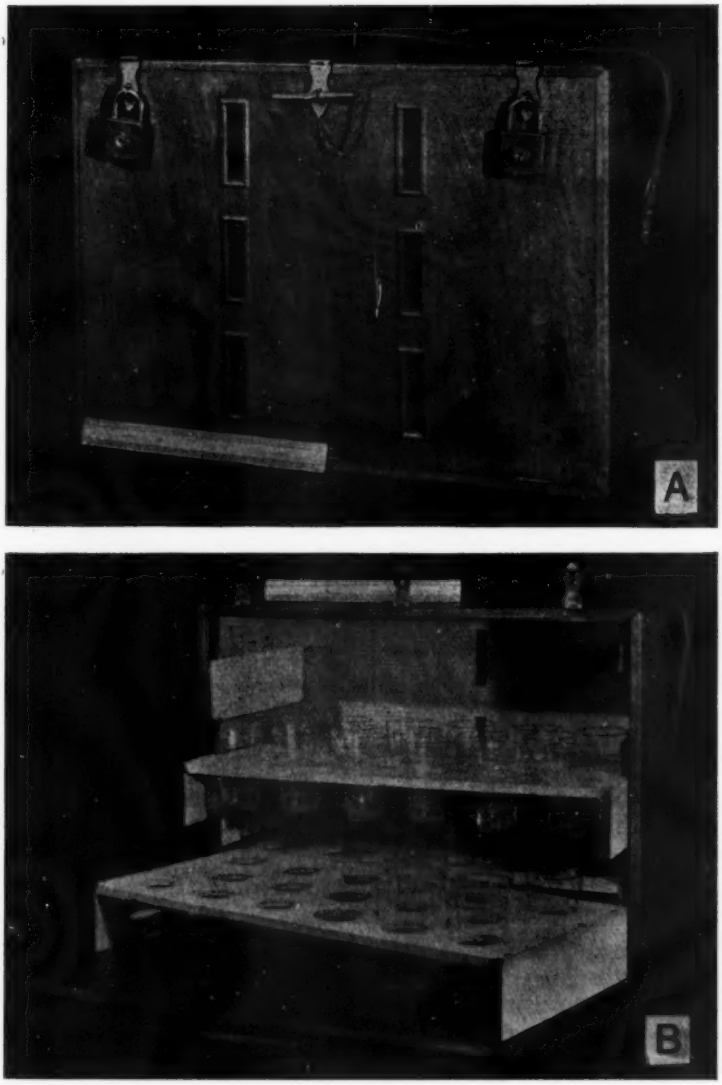

Fig. 1. Portable and flyproof breeding cage. A, closed; B, opened.

the case was found to contain no foreign eggs or larvae, the exposed beef being uncontaminated. All the cultures were reared out, and were found to have retained their purity. As an additional precaution during subsequent use, the box was kept indoors and in a dark place, and was examined within and without daily, as was each separate jar and cover. This case may be used for a variety of purposes by removing the sliding shelves, or by replacing them by plain shelves or pigeonholing made to fit. The entire weight of the apparatus when all the jars are full is 50–60 lb.

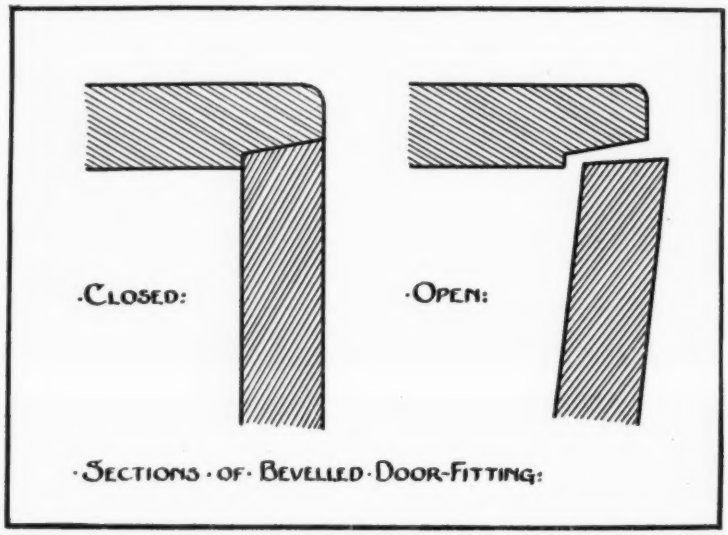

Fig. 2. Sections of door-fitting.

PREVENTIVE MEASURES

Most of the farms concerned were of the small general type, none dealing exclusively with sheep. In few of them did the flock number more than 500. In four only were whole-time shepherds employed; in the remainder the sheep were supervised either by the farmer himself, or by a farm worker, in both the latter cases the general work of the farm occupying much of the day. In every case the sheep were examined at least once daily, by the individual concerned, during the fly season. The flock was invariably scattered over wide tracts of comparatively rough ground, rendering such supervision arduous and lengthy. Seldom could the shepherd be perfectly certain that no sheep had been overlooked—an important point when the amount of damage done by maggots in a single day is considered.

None of the preventive measures in use in the districts concerned can be considered adequate in dealing with the pest. As the present dipping regulations are concerned with the prevention of sheep-scab rather than of maggot, the

first compulsory dipping of the year comes, in most districts, well after the beginning of the fly season. There is no doubt that in the majority of cases an earlier dipping would go far toward the prevention of these early cases which are so liable to progressive restrike throughout the summer. Such dipping as does take place is seldom efficiently carried out. We have observed a widespread tendency toward the use of over-strong solutions, with consequent skin irritation, loss of condition, and liability to strike. The time of immersion is usually much below the minimum prescribed by the maker of the dip. The majority of the troughs and pens are of the most primitive nature, and, a point of great importance, are rarely, if ever, cleaned. Two cases were noted where the dipping trough was never washed out, fresh dip merely being added to the existing fluid when the farmer considered this necessary. Few farmers realize the danger of overcrowding the sheep in small and dirty pens and drippers, with consequent fouling of the wool—a potent predisposing factor in strike. The conscientious use of recognized sulphur-arsenic dips, freshly made up at the correct strength, or even under strength, in clean troughs and with roomy dripping pens, have been found to be effective for a much longer time.

Where maggots are actually present on a sheep, many of the methods of removal are too drastic, causing inflammation and even the localized loss of wool. While many measures, such as the application of dilute dip, whale-oil and sulphur, Stockholm tar and the proprietary maggot-oils are satisfactory, we have often observed the unfortunate results produced by the use of concentrated carbolic and arsenical solutions or of crude paraffin. Among other substances used are dry earth, flowers of sulphur and sodium bicarbonate. Whale-oil and sulphur ointment, while forming a protective coagulum effective in preventing the irritation of open wounds by small muscids, anthomyids, etc., is of little use as a deterrent to maggot-flies. Owing to its property of cracking after drying and thus of damaging the underlying scab, it provides ready-made routes of entry to the already damaged tissues, nor does it seem to have a markedly harmful effect on the maggots themselves. Furthermore, healing is often retarded by the tendency of the sheep to bite at such dressings. While most farmers realize that maggots too rapidly killed *in situ* and not properly removed from the fleece will remain there rotting, and so attracting flies, most fail to appreciate the fact that living larvae rubbed out of the wool are, in many cases, able to complete their metamorphosis. Many shepherds, not wishing to kill the maggots in the wool, rub them out before applying the remedy, thus permitting a large number to escape. The advantages of clipping closely the wool round maggot wounds cannot be overemphasized; this process, besides rendering fresh egg-masses more obvious, seems to lessen the tendency to reinfection.

There is no question that indirect measures of control, when properly carried out, may be most effective, though this fact is seldom fully realized by those concerned. The process of "crutching", i.e. of shearing closely the breech and hindlegs down to the hocks, of clean and dirty sheep alike, as opposed to

the usual method of clipping scouring sheep only, has been advocated (Robinson, 1935). We have seen this system employed with distinct advantage on two farms. The proper disposal of dead sheep and other carrion is of the highest importance as an indirect method of control. On very few farms is this effectively carried out. A common practice in the West Highlands is to allow the carcass to lie till the wool can be plucked off it—usually a period of some days. It is by this time, naturally, swarming with larvae, and the subsequent perfunctory burial, if any, in about 1 ft. of earth assists rather than hinders their development, as has been shown by Fuller (1932). Over the whole area the practice of allowing carcasses to lie exposed is prevalent—five such carcasses have been counted in the course of a mile's walk—most farmers firmly believing that the eventual burial destroys the maggots. In this connexion it is interesting to note a case recorded in the Press during the summer of 1935: some 200 sheep reported stolen from an area in Mid-Argyll were almost all eventually found by the police, not stolen but dead, mainly of myiasis—many of the carcasses being so infested as to be visibly moving, while a low, bubbling noise was audible from some little distance. On two farms only have we seen the treatment of carcasses with poisons before burial. Nowhere have we found any attempt to deal with small carrion, rabbits, birds, etc. A factor of importance and entirely outwith the farmer's control is the scandalous condition of many country slaughter-house "middens", where huge mounds of offal lie exposed and accumulating over long periods—ideal breeding grounds for large numbers of blow-flies. It is probable that such a state of affairs, favouring, as it must, the development of a carrion-fly population far too great for the available normal food supply, forces the insects concerned to seek new breeding places, such as living sheep. In this connexion it may be emphasized that many authorities, such as Froggatt (1904, 1915), attribute the first serious spread of sheep myiasis in Australia to a similar cause, namely, to the exposure of multitudes of carcasses as a result of the extensive campaign against the rabbit pest in that country. While in Britain the presence of large rabbit warrens will undoubtedly stimulate blow-fly increase owing to the number of bodies in their vicinity, we believe that their importance in aiding the over-wintering of flies has been exaggerated, definite evidence indicating that blow-flies pass the winter not as adults or pupae, but mainly as hibernating larvae. This point will be considered in greater detail below. Of recent years a few enterprising farmers have adopted the trapping system. We have noted two farms where the "Western Australian" trap as modified by de Vries (1933) is in use, and a few where bait is suspended over pails of sheep dip or lime, apparently with good effect.

Seasonal incidence

While the fly season proper may be said to extend from the middle of June to the middle of September, various workers have recorded cases much earlier or later in the year. MacDougall (1909) noted cases in every month of

the year except December, January and February, and Walton (1918) has found sheep attacked as early as March and April. In Ayrshire the pest seldom occurs before June. In Argyllshire we have records of sheep maggot in the first week of April, and have observed many cases in mild weather from the beginning of May onward. We have not noted the occurrence of myiasis in the districts concerned after the beginning of October.

The weather conditions influencing strike seem to be fairly constant—as noted by most workers, the flies attack most readily in warm, moist weather, while cold, wind and rain are unfavourable. On warm bright summer evenings, oviposition may occur at a comparatively late hour, on one occasion as late as 9 p.m. (British Summer Time) at the end of July. The mild winters and hot summers of 1932–3–4 have definitely contributed to the spread and increase of sheep myiasis in Scotland. As opposed to this, the cooler summer of 1935, though preceded by a winter as mild as the above, has shown, if anything, a decrease in the severity of maggot-fly attack in the districts studied.

TYPES OF COUNTRY

Little or nothing can be added to the existing accounts of the relation between sheep myiasis and the ground conditions involved. These have been fully discussed by Walton (1918) and others. We have found it a fairly general rule that the flies prefer low ground—sheep on high heathery country being less liable to attack. While avoiding the shade of woods, the flies seem to be most prevalent in sheltered spots, especially where much bracken is present. It is hard to overrate the importance of the latter as a predisposing factor in myiasis. Flies shelter in large numbers in bracken during heat—a situation much sought by the sheep also, for the sake of shade and coolness. In wet and cool weather, and by night, it acts as an efficient protection to many insects which would otherwise succumb. Equally important is the tendency of struck sheep to leave the rest of the flock and lie down in dense clumps of bracken, from which, in their peculiarly apathetic condition, they refuse to be dislodged by dogs. They are thus liable to be overlooked by the shepherd till they are past recovery, the carcasses of such victims remaining as hidden and unsuspected breeding grounds. It has been recognized by several authorities that wet sheep are particularly liable to attack, probably owing to the distinct odour of moist wool. Bracken remaining damp, as it does, long into the heat of the day, wets the neck and shoulders of sheep forcing their way through it, and thus renders these parts more attractive to blow-flies. On many farms the bracken, uncut from year to year, lies on the ground as a dense protective covering, in which a certain slight degree of warmth is probably generated by slow fermentation. Ground covered by such a layer is protected from extreme cold, and is less liable to freeze. This action is probably of importance in aiding the survival of hibernating prepupae during adverse winter conditions. On one Argyllshire farm we have noted a very substantial decrease in sheep maggot coincident with the cutting of large areas of bracken.

Damage done to sheep

On all the farms concerned the flock consisted of Blackface or cross-bred strains or both, sometimes a few Border Leicesters also being present. We have never obtained maggots from any of the last-named variety. On none of the farms studied were Cheviots stocked, but Cheviot owners state that this breed readily fall victims to blow-fly attack. In the west the Blackface is generally considered to be the most resistant variety.

With regard to the damage done to the sheep themselves, while it is known that any part may be affected, we have noted the high percentage of breech or tail strikes, mainly, but by no means always, on scouring sheep. This type of infection occurs mostly among ewes, lambs seeming more liable to be struck on the flanks and back. Rams seem to be most susceptible to attack on the neck and shoulders where the wool is thick. The horns of rams also seem to be peculiarly liable to infestation, especially if there has been any ulceration round the base, the face also being attacked in cases where there is friction between the horns and the cheeks during mastication. Wethers castrated by methods involving opening of the scrotum are frequently victims of "purse-strike" unless the wound has been carefully and completely sewed up. Maggots frequently occur where foot-rot is present, actually being introduced by some farmers with the object of clearing out the disease—a case of such treatment recently appeared in Court on a charge of cruelty. Beyond doubt this method is effective in clearing up the condition, but the larvae, unless removed in time, show a well-marked tendency to migrate up the leg and attack healthy tissue. As noted by Ratcliffe (1934) myiasis of the flanks is often induced by the soiling of the wool of this region by dirty or diseased trotters when the animal is lying down.

The majority of primary superficial lesions observed have been clean wounds, which, if not unduly disturbed, generally heal rapidly by granulation. If, however, as is often the case, the wound is much handled during treatment, there is definite danger of infection by pyogenic bacteria, especially by *Bacillus pyocyaneus* (*Pseudomonas aeruginosa* Migula), probably the commonest species in wool. The consequent formation of a discharging ulcer renders the wound extremely liable to restrike, with the added danger that the larvae, carrying the infection into the deeper tissues, may set up a fatal septicaemic or pyaemic condition.

Strike and life history

While absolute knowledge is as yet wanting as to the exact reasons for primary strike, several important predisposing causes have been recognized and, in addition to those already discussed, the following are important. It has been demonstrated by Holdaway (1932) that the main initial cause of body strike is bacterial activity in the wool. This, while obviously at a maximum where urine or faeces have soiled the fleece, also occurs in regions apparently clean and unaffected, and is the most probable explanation of strike in such

parts. Where the wool is kept moist by water or sweat the extreme activity of the bacteria produces decomposition of the fibres or "wool-rot". This condition is known to be attractive to maggot-flies. It has been shown experimentally by Freney (1932) that artificially decomposed wool fibre (keratin) is lastingly attractive to the primary Australian blow-flies (including *Lucilia sericata*) to such an extent that it forms an excellent bait for traps. These facts show how warm, wet weather, hard driving or improperly trained dogs may, by wetting the wool, or causing the animals to sweat, stimulate bacterial growth and wool degeneration, and thus pave the way for maggot-fly attack.

Larvae in wool may either remain on the skin surface, causing comparatively little damage except to the fleece, or they may bore inward with more serious consequences. The method adopted seems to depend, in many cases, on the number of maggots present, and on the condition of the fleece. Larvae in small numbers seem to be able to obtain adequate nourishment from wool-yolk and from the superficial layers of the skin. This is seen mainly in the earlier parts of the year before the strikes are numerous. Such maggots differ in appearance from those which bore deeply, owing to the colour of the contents of the dorsal food reservoir—a fact which, together with their behaviour, has probably given rise to the widespread but erroneous belief that they belong to a different species, particularly active in late spring, *Calliphora* generally being the supposed agent. It follows from the above facts that a sheep with long, thick wool is more liable to exhibit this superficial type of myiasis, especially in the earlier months. Lambs and shorn sheep, on the other hand, though less liable to attack, usually suffer a much more extensive invasion of the tissues when struck. Larvae, when present in large numbers, almost invariably burrow more deeply, usually with serious consequences. Depending upon the part of the body struck, they may either penetrate at once, or may migrate some distance in the search for a soft or more easily invaded part, such as the flank or the fleshy part of the haunch. We have noticed that such migrating larvae usually tend to travel upward or forward, according to their original position. In hot weather, and under suitable conditions, hatching and larval growth may be greatly accelerated—in two cases the larvae had reached the third instar in a single day—one of these will be described below. Ratcliffe (1934) describes larvae which attained full growth in 48 hours at 90° F., the approximate temperature of sheep's wool near the skin. It has been stated by various workers that *Lucilia* larvae are not coprophagous. In several cases, however, they have been observed feeding on dung in wool, and, in one instance, a large batch were reared from the egg to maturity on such a diet alone, the larval period being much protracted and the adults small though perfect. In this connexion it is interesting to note that Bogdanow (1928) has reared eleven generations of *Calliphora erythrocephala*, normally a carrion feeder, on an exclusive diet of human faeces.

It has invariably been our experience that under natural conditions the

winter is passed in a hibernating prepupal phase. On several occasions mature larvae of several species have been kept throughout the winter under a variety of natural and unnatural conditions, outside and indoors, pupation never taking place till April or May. In this particular we are entirely in agreement with Maldwyn Davies (1934). We consider the supposition of some authors that the winter is passed in the pupal state as most unlikely, nor have we seen adequate evidence adduced in its support. In no case have we found that the blow-flies can survive as pupae for more than 3 or 4 weeks. This fact is borne out by the following observations. It has been found possible to induce pupation in hibernating prepupae by one method only—continued exposure to high temperatures in hot houses. This is actually the method employed to hasten the emergence of autumn larvae, and so to reduce the high mortality prevalent among those permitted to hibernate normally. Pupation having been induced by such methods, it was found impossible to prolong its duration by any such means as re-exposure to normal winter temperatures, either inside or outdoors; under such conditions the adults either emerge after a pupal period of normal length or die within the puparium. The pupa, far from being a resistant instar as is usually supposed, has been found to be highly vulnerable, and susceptible to changes in temperature, humidity, etc., which are not injurious to the mature larva or prepupa, these stages being unaffected by relatively great variation in these factors. The prepupal phase can be prolonged almost indefinitely and is thus well adapted for hibernation.

We have never found parasites of any description in cultures of larvae taken from living sheep. This point has been noted by Maldwyn Davies (1934).

Species of fly involved

It has been demonstrated by many workers that *Lucilia sericata* Mg. is almost exclusively the sheep maggot-fly concerned in Britain. Cases of attack by other flies have, however, occasionally been recorded. MacDougall (1909) mentions three cases of infection by *Calliphora erythrocephala* Mg. and three of the same species and *Lucilia sericata* mixed. In the same paper, reference is made to strike of sheep by *L. caesar* in Huntingdonshire and Kent, Theobald being the authority quoted. Hewitt (1914) mentions *L. caesar* as a sheep blow-fly. Walton (1918) states that *Calliphora erythrocephala* undoubtedly causes trouble, but gives no evidence. Maldwyn Davies (1934) notes two mixed infections of *Lucilia sericata* and *Calliphora erythrocephala* (the latter "in small numbers"). Ratcliffe (1934) states that *C. erythrocephala* is known to attack sheep. In the present investigation we have been concerned in an attempt to show that other species than *Lucilia sericata* do occur, in certain districts at least, as secondary agents of sheep myiasis following on or coincident with attack by this species, and that they may infest wounds and sores produced, in the first instance, either by disease or by larvae. The evidence indicates that these flies do not initiate myiasis unless some such conditions as foot-rot

or a purulent sore be present. All the species involved are normally carrion-flies. We have found them almost exclusively in heavy or prolonged infections of *L. sericata*, in old wounds, etc. While the small numerical proportion of these flies, when present, and their rarity or total absence in myiasis in certain districts indicate clearly their occasional and secondary role, and render them, as yet, of minor importance, their presence leads to the belief that they may be following the world-wide parasitic trend of the Calliphorinae.

The flies concerned will now be considered briefly: *L. caesar* (L.) is a common carrion-fly over the entire area; we find it to be the most important of these secondary species and have recorded it in twenty-four cases of myiasis. *L. illustris* Mg., though not so common, is fairly well distributed in the west of Scotland. While in the past some workers have not recognized this species as distinct from *L. caesar*, the structure of the male genitalia as demonstrated by Séguy (1928) and Aubertin (1933) has placed the point beyond doubt. We have noted three cases of infection by this fly. *Calliphora erythrocephala* Mg., though one of the commonest species, and widely believed to cause sheep myiasis, we have found five times only. *C. vomitoria* L., though very common in some of the districts studied, has been reared from only two cases of maggot infection. *Protophormia terraenovae* R.-D. (=*groenlandica* Zett.) is a widely distributed British carrion-fly known by a variety of names such as *Phormia terraenovae*, *Protocalliphora groenlandica*, etc., the synonymy giving rise to much confusion. We have employed the above name (as given by Bezzi, 1922, and Séguy, 1928) after careful consideration of the evidence of morphology and priority. We have reared it in seven cases of sheep myiasis. In three instances a single larva of *Muscina pabulorum* Fallen, easily recognized by the characteristic posterior spiracles, has been taken from a living sheep and reared to maturity. In several of these cases we have found more than one of the species concerned occurring along with *L. sericata* on a single sheep. Fuller details are given below.

While cases of infection by the above-mentioned species of *Lucilia* and *Calliphora* are not altogether unexpected or unprecedented, especially in sores or wounds, the occurrence of *Protophormia* may occasion some surprise. In America, however, the closely allied species *Phormia regina* Mg. is well known as a sheep blow-fly in Texas (Bishopp, 1915), California (Herms, 1915), and British Columbia (Hearle, 1933). The presence of *Protophormia terraenovae* may be diagnosed by its characteristic habit of pupating in the fleece of the living sheep instead of in the ground. The puparia are readily identifiable by means of their well-developed lateral ridges and terminal processes—features sufficiently prominent to be recognized by a few observant farmers. We have seen these large and distinctive puparia on living sheep in various localities, but so far have reared the adults from larvae on sheep in the case of two farms only. The puparia become most evident on shearing, when they are sometimes found in fairly large numbers in the thick wool close to the skin.

The identification of species of *Lucilia* other than *L. sericata* has been confirmed in each case by microscopic examination of the genitalia. Miss D. Aubertin, of the British Museum, was kind enough to identify our first specimens of *L. illustris*.

Comparison of areas with rearing records, etc.

Within the area studied, a variety of conditions was encountered both in the type of country and in the methods of farming. The Ayrshire farms are all situated about 12 miles inland, at heights over 700 ft., round Fenwick Moor, a wide tract of rough grassland with little bracken. They lie on the borderline between rich arable and dairy-farming country and undulating moor. Farming in this district is of a high standard. The Arran farms, all close to the sea, are fairly low-lying. The ground is rough and is heavily covered with bracken and some heather. The remaining farms, in Argyllshire, like those in Arran, lie close to the sea, at varying heights. The country is very broken, and supports a dense growth of bracken and heather, and, in places, hazel scrub, etc. In Argyll farming is of a simpler nature than in Ayrshire.

The occurrence of the secondary flies was found to be related approximately to the above conditions. Comparatively rare in Ayrshire, their incidence increased in the western districts, reaching a maximum for the area in Kintyre and Knapdale. Their occurrence was obviously dependent on the type of country, efficiency of supervision, relative abundance of the species concerned, and local prevalence of myiasis which is much more severe in Argyll than in Ayrshire.

Below are given separate accounts of the various farms studied, with records of each case in which flies other than *L. sericata* were met. To save space no precise record is given of the many cases in which *L. sericata* alone was reared:

(1) *Sheep maggot in Ayrshire* (R. C. M. Thomson)

The farms to which particular attention was paid are all situated on the same type of moorland country, as described above. From the present point of view, the most important features of the ground are the open, undulating nature of the rough grassland, the absence of deep, sheltered glens, and the relative scarcity of bracken. In Ayrshire, as in many other parts of Scotland, there is a widespread belief that there are two distinct kinds of maggot or "mawk" associated with two different flies. In the early summer there is a "soft" "dung" or "black-headed mawk" supposed to be that of *Calliphora*, which is replaced later in summer by the more harmful "red-headed mawk" associated with *L. sericata*. Breeding records show that this hard and fast distinction is quite unfounded. *Calliphora* has not been recorded as a sheep maggot-fly in this area, and "black-headed mawks" of various different types have all proved to be those of *Lucilia sericata*. *Protophormia* has only been taken from sheep on one occasion, but all farmers and shepherds have, at

some time or other, seen the black puparia in the wool of living sheep. Although widespread, it is evidently only of occasional occurrence on sheep. The secondary sheep maggot-flies play a less important part in Ayrshire than in any of the other areas investigated. Breeding records have shown that the vast majority of strikes are due to *Lucilia sericata*, and numerous cases of attack by this species alone have been obtained from the following farms: Craigends, Tayburn, Low Rushaw, Muirside, Blackbyre, and Cairnhill. Secondary sheep maggot-flies have been recorded in the following few cases:

Drumtee Farm. This is situated on a bleak tract of rough grassland, merging with moorland, near the north boundary of Aryshire. Sheep maggot is less severe here than in other farms of the area.

5. vii. 33. Several pupae and full-grown larvae of *Protophormia* as well as a single larva of *Muscina pabulorum* were taken from the wool of a sheep. This was evidently a secondary attack in which the primary larvae of *Lucilia sericata* had left the sheep in order to pupate, and progressive restrike had been checked by unfavourable weather.

Craigends Farm lies in a hollow in the moor, and the ground, though rough, is less exposed than at Drumtee.

10. vi. 33. Larvae of different sizes were taken from a mild case on the soiled hindquarters of a Blackface lamb. This was an unusually early strike for this farm. The flies recorded were:

L. sericata	...	22
L. illustris	...	7

Tayburn Farm is situated near Craigends, and has very similar ground and exposure. None of the several cases examined here during the summer was serious, and with a single exception they were found to be caused by *L. sericata* alone.

16. vii. 35. Several larvae were taken from a small patch on the hip of a Blackface lamb. The flies bred were:

L. sericata	...	23
M. pabulorum	...	1

Muirside Farm is situated on very exposed, undulating moorland and the sheep are over a much wider area than in the other farms. Maggot-fly was very troublesome here during the summers of 1933–4–5, but, owing to strict supervision, was kept well under control. At one time in July 1935 only four sheep out of 500 were struck. Several cases of attack by *Lucilia sericata* alone were recorded during the summer, and in only one instance did secondary flies appear.

29. vii. 35. The flies recorded were:

L. sericata	...	42
L. illustris	...	4

(2) *Sheep maggot in Arran* (R. C. M. Thomson)

In the summer of 1935 an investigation was carried out in three localities in Arran—Brodick and Lamlash on the east coast, and Machrie on the west coast. Sheep farming on a large scale is confined to these three districts, and the conditions obtaining there may be taken as fairly representative of the whole island. Here, as in most other places in Scotland, sheep maggot has become increasingly serious in the last few years, and has spread to the south of the island, where, till recent times, it was unknown. The farmers and shepherds of six farms recognize three different "mawks" as compared with

the two of Ayrshire, and associate them with three distinct flies, *Calliphora*, *Protophormia*, and *Lucilia*. Only the two latter species, however, have been recorded from these farms, and *Calliphora*, if it does occur at all, must be a very rare sheep maggot-fly in this area. All those concerned with the actual handling of sheep have seen the black puparia of *Protophormia* in the wool of living sheep, and, although it has not occurred in any of the breeding

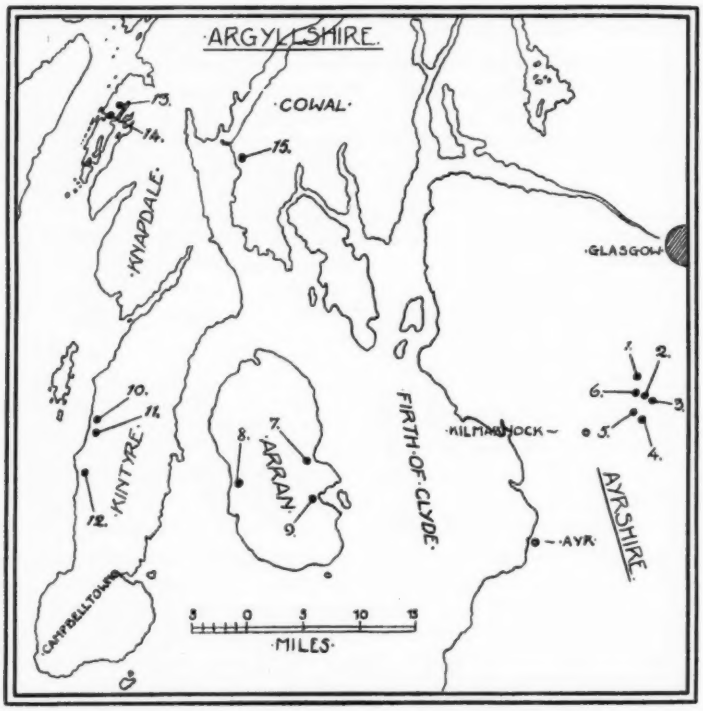

Fig. 3. The collecting area. Names of farms: 1, Drumtee; 2, Tayburn; 3, Craigends; 4, Muirside; 5, Low Rushaw; 6, Blackbyre; 7, Glencloy; 8, Machrie; 9, Glenkill; 10, Beachmeanach; 11, High Crubasdale; 12, Barr Mains; 13, Arinafadbeg; 14, Carsaig; 15, Fearnoch.

records for this district, it is evidently a fairly generally distributed, if never abundant, sheep maggot-fly. Larvae were obtained from three farms, and the separate records are considered in more detail below:

Glencloy Farm. This farm is situated in a long, narrow glen, running down to the sea, and is protected on three sides by steep hills. Owing to the sheltered position, the glen is very hot in summer, and this, together with the rough nature of the ground and the general covering of dense bracken, make it particularly difficult for the shepherd to keep the maggot-fly under control.

9. viii. 35. Larvae of various sizes were taken from an extensive, open wound along the

back of a Blackface ewe which had previously been attacked and subsequently cleaned of maggots. The flies recorded were:

L. sericata	...	31
L. caesar	...	47

Glenkill Farm. This farm is situated near the sea at the beginning of a wide glen. The ground, though rough and bracken-covered, is much less sheltered than at Glencloy.

8. viii. 35. Larvae of different sizes were taken from a mild case on the tail region of a Blackface ewe. The flies bred from these were:

L. sericata	...	59
M. pabulorum	...	1

Machrie Farm lies at the edge of a wide stretch of flat country on the west coast of the island. The ground and exposure are very like those of the west of Kintyre, and the slopes of the hills are similarly heavily covered with bracken. Sheep maggot was not so severe as in Glencloy, but continued much later in the season, in spite of cold, wet weather. Three samples of larvae were obtained, all of them yielding small proportions of *Lucilia caesar*.

9. viii. 35. The flies recorded were:

L. sericata	...	116
L. caesar	...	1

11. ix. 35. The flies recorded were:

L. sericata	...	68
L. caesar	...	8

17. ix. 35. The flies recorded were:

L. sericata	...	41
L. caesar	...	6

(3) *Sheep maggot in Argyllshire* (A. J. Haddow)

In the districts studied, the main enemy of the farmer is probably the dense growth of bracken, which, together with the moist, mild climate, favours the increase of sheep blow-flies. Myiasis of sheep in this area is probably as serious as anywhere in Scotland, fatal cases being not infrequent, and heavy infestations an everyday occurrence. The shepherd's task is rendered difficult by the fact that he is usually expected to perform other duties besides care of the flock. The country being rough, the difficulties of supervision are great. At Beachmeanach, *Protophormia* is abundant, and from this farm only has it been recorded as a maggot-fly. The puparia have however been observed in wool at Cleit, West Kintyre. On Carsaig Farm, Knapdale, an effective campaign against sheep maggot during 1935 resulted in a reduction of fatalities by over 90 per cent as compared with 1934. The total number of strikes was also very substantially reduced. The measures employed were: cutting of bracken, building of large pens, use of an isolation field for bad cases, regular use of clean and properly mixed dip, crutching, prompt carcass burial, daily examination of the flock by an efficient shepherd, and immediate and careful treatment of all struck sheep. Carcass burial elsewhere was usually rather tardily and inefficiently done. Many Argyllshire farmers firmly believe that young larvae of *Lucilia sericata* belong to different species from the fully

grown ones. Below is given a record of cases in which flies other than *L. sericata* were reared:

Beachmeanach Farm, West Kintyre, consists of a stretch of flat shore fields with dense tracts of bracken and couch grass, an intermediate zone of hill pasture with much bracken and whin, and a considerable area of heathery hillside. The sheep, which are kept mainly on the high ground, gather in hot weather in a narrow, bracken-choked glen. In spite of careful dipping and supervision, maggots cause much trouble on this farm. Blow-flies of many species are peculiarly abundant here.

3–11. vii. 34. Larvae from various cases were reared in rather limited quarters, with consequent high mortality. The following emerged:

L. sericata	3
Pr. terraenovae ...	16

30. vi. 35. Larvae in large numbers and all stages were taken from a large wound on the haunch of a ewe. The fleece was much soiled. There was a considerable bare area on the flank. The following emerged:

L. sericata	55
L. caesar	2
Pr. terraenovae ...	1

5. vii. 35. Considerable numbers of maggots in all stages were found in a wound 3 in. square on the haunch of a ewe. The wound, which was necrotic, penetrated almost to the bone. The flank and back were also infested. This case terminated fatally. The following emerged:

L. sericata	23
Pr. terraenovae ...	13

7. vii. 35. Larvae were collected from a heavily infested ewe. They were present in vast numbers all over the back and the left haunch and flank. There was most extensive destruction of the superficial tissues. The fleece in these regions was hanging in loose strands. The following emerged:

L. sericata	1178
L. caesar	54
Pr. terraenovae ...	9

8. vii. 35. An old ewe was found to have a maggot wound extending from the hock upward to the middle of the back. The larvae were mainly in the first stage. The following emerged:

L. sericata	225
L. caesar	12
Pr. terraenovae ...	1

10. vii. 35. Larvae in moderate numbers and all stages were taken from the haunch of a hogg. The skin was bare but unbroken. The wool was much soiled with urine and faeces. The following emerged:

L. sericata	248
L. caesar	7
Pr. terraenovae ...	18

10. vii. 35. Both haunches of a ewe were found to be covered with larvae, mainly in the first stage. The skin was bare but unbroken. The wool was matted with urine and faeces. The following emerged:

L. sericata	246
L. caesar	14

27. vii. 35. An old maggot wound in the flank of a ram was found to be full of eggs and first-stage larvae. Clean wounds were also present on the other flank and both sides of the neck. The wounds were disinfested, clipped and treated with dip. The next day the same wound and a new one below it were surrounded by eggs and swarming with larvae *already*

in the third stage (i.e. within 1 day). The following day the wounds were infested, and a new one was present between the shoulders. The following emerged:

L. sericata	...	...	171
L. caesar	...	...	4

High Crubasdale Farm, West Kintyre, is situated at a height of 300–400 ft. on an exposed, heathery hillside. Sheep blow-flies are not a serious problem here. The stretches of pasture are fairly clear of bracken.

6. vii. 35. Moderate numbers of third-instar larvae were collected from the tail and haunches of a scouring ewe. The skin was unbroken. The following emerged:

L. sericata	...	...	7
L. caesar	...	...	14

6. vii. 35. A few third-stage maggots were found on the tail of a scouring ewe. The skin was unbroken. The following emerged:

L. sericata	...	...	13
L. caesar	...	...	1

Barr Mains Farm, West Kintyre, closely resembles Beachmeanach (see above). It is, however, much more exposed, and the hill-pasture zone is rather marshy, with extensive reed-beds. The farm is divided by a deep glen, containing much bracken.

22. ix. 34. Two very small egg clusters were taken from the margin of an old, healed maggot wound on the haunch of a clean lamb. The following emerged:

L. caesar	...	...	22
C. erythrocephala	...		4

23. ix. 34. Four small groups of larvae were taken from wounds between the hindlegs of a scouring ewe. In consequence of previous attacks, most of the wool of the back was missing. The larvae were all in the first and second instars. The following emerged:

L. sericata	...	...	58
L. illustris	...	...	28

23. ix. 34. Three clusters of eggs were removed from the haunch of a clean lamb. The following emerged: *C. erythrocephala* ... 500 approximately.

Carsaig Farm, North Knapdale, occupies a narrow glen. The floor of the glen is sheltered and marshy; the walls are covered with abundant bracken and heather. Pasture is rather scanty. There are very few trees.

4. viii. 35. Maggots in large numbers were taken from a wound of 3 in. diameter on the haunch of a clean wether lamb. The skin was rendered spongy by multitudes of small holes. The surrounding fleece was also heavily infested. The following emerged:

L. sericata	...	...	23
L. caesar	...	...	84

27. viii. 35. Moderate numbers of larvae in all stages were taken from the trotter of a ewe suffering from foot-rot. There had been extensive penetration of the diseased part. The following emerged:

L. sericata	...	...	1
L. caesar	...	...	161
C. erythrocephala	...		2

A few clusters of eggs were collected from the flank of the same ewe, at a point which would be in contact with the affected trotter when the animal was lying down. The following emerged:

L. caesar	...	...	202
C. erythrocephala	...		1
C. vomitoria	...		1

Arinafadbeg Farm, North Knapdale, occupies a steep hillside. The higher parts are

heathery. Below is a belt of hill pasture, with dense tracts of bracken, hazel scrub and pines. The low ground consists of sheltered fields surrounded by woods of hazel and pine. The whole is fairly sheltered. Sheep maggot is very troublesome on this farm.

29. v. 35. A large number of larvae in all stages were found feeding close to the skin on the haunches of a scouring ewe. The following emerged:

L. sericata	441
L. caesar	21

30. v. 35. A large number of larvae were removed from the hindquarters of a ewe. The fleece was wet with urine. The tail was much damaged. The following emerged:

L. sericata	159
L. caesar	7

12. viii. 35. The base of a ewe's horn was found to be full of maggots. They had penetrated within and around the horn, which was rather loose. The wound was discharging. The following emerged:

L. caesar	51

12. viii. 35. A large area above the tail of a scouring ewe was found to be infested with small larvae, which were just penetrating the skin. The following emerged:

L. sericata	4
L. caesar	71

27. viii. 35. Vast numbers of larvae were taken from a weakly wether lamb. The head, neck, shoulders, hindquarters and belly were swarming with larvae, many thousands being present. The case terminated fatally the next day. There was a heavy mortality in the cultures. The following emerged:

L. sericata	10
L. caesar	143
C. vomitoria ...	22

28. viii. 35. The cheek and ear of a wether lamb were found to have been almost totally destroyed by maggots. There had been extensive invasion of the deep tissues. A foul discharge was present. The lamb died a few hours later. The following emerged:

L. sericata	13
L. caesar	97

Fearnoch Farm, Kilfinan, Cowal, consists of heathery hill land, with dense belts of bracken, a few flat fields, and some woods. During 1933–4 sheep myiasis was prevalent, but few cases occurred in 1935.

17. viii. 35. A moderate number of larvae were taken from above the tail of a scouring ewe. The skin was just breaking. Much blow was present. The following emerged:

L. sericata	39
L. caesar	14

(4) *Farms outwith the area* (A. J. Haddow)

Material from Wester Ross and from Gleneffock, Angus, consisted of *L. sericata* alone.

Kirkton of Crawford, Lanarkshire.

25. ix. 34. A batch of larvae, taken from a single sheep was sent by hand. Approximately 200 *Calliphora erythrocephala* emerged.

Auchmull Farm, Glenesk, Angus, is situated in a fairly wide, grassy glen, with much bracken and some trees.

4. vii. 35. A consignment of larvae was received by post. The following emerged:

L. sericata	1026
L. caesar	9

SUMMARY

1. Sheep myiasis was investigated in certain districts in Ayrshire, Arran and Argyllshire.

2. Larvae from cases of myiasis were collected and bred out in flyproof apparatus.

3. The fly season was found to last approximately from May till September, reaching its height in July and August.

4. The western districts were found to be the most heavily affected.

5. The flies were found to be partial to low-lying, sheltered ground, especially where much bracken was present.

6. The methods of carcass burial were found to be primitive and inadequate. In most of the farms dipping was carried out without sufficient attention to cleanliness and adequate time of immersion.

7. The hibernation of various species of blow-fly was studied. They were found to pass the winter as prepupae, pupation never occurring normally till April or May.

8. Several species of carrion-feeding blow-fly besides *Lucilia sericata* were found to be causing secondary myiasis, occasionally and in small numbers, in certain districts, mainly in the west. The species involved were: *Lucilia caesar*, *L. illustris*, *Protophormia terraenovae*, *Calliphora erythrocephala*, *C. vomitoria* and *Muscina pabulorum*. Four of these species *Lucilia illustris*, *Protophormia terraenovae*, *Calliphora vomitoria* and *Muscina pabulorum* have not previously been recorded from sheep. Records of cases in which these flies occurred are given.

We wish to thank Dr R. A. Staig of the Department of Zoology, University of Glasgow, for valuable help and advice, Mr W. H. R. Lumsden for much practical assistance and for material from Angus and Wester Ross, and, above all, the farmers and shepherds whose friendliness and co-operation have enabled the work to be carried out.

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(*MS. received for publication* 22. II. 1936—Ed.)

A NEW COCCIDIAN, *WENYONELLA HOAREI* N.SP. FROM AN INDIAN SQUIRREL

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(With Plate VII, containing Figs. 1–19)

In June 1935 we observed in the intestine of an Indian squirrel, *Sciurus* sp.[1], a coccidium which, since it has tetrazoic tetra-sporocystid oocysts, must be placed in the genus *Wenyonella* recently created by Hoare (1933).[2]

The type species, *Wenyonella africana* Hoare, was found in the small intestine of an African snake, *Boaedon lineatus*. Endogenous stages were scanty, and, since he could not distinguish the gametocytes with certainty and saw no microgametes, Hoare was unable fully to justify his opinion that *Wenyonella* should be placed among the Eimeriidae.

Since we have found abundant endogenous material in the epithelium of the small intestine of one of our three infected squirrels and are in a position to confirm Hoare's assumption satisfactorily, we think it worth while to publish observations additional to those in our preliminary note (Ray and Das Gupta, 1935). Our coccidium differs in certain particulars from *W. africana*, and we regard it as a new species, which we have named *W. hoarei* n.sp., after Dr Hoare.

Wenyonella hoarei n.sp.

Our observations were made partly on living material, and partly on smears and sections, fixed in Schaudinn's fluid or in Bouin-Duboscq-Brasil, and stained with Heidenhain's iron-haematoxylin or Delafield's haematoxylin. For sections we also employed Mallory's triple stain and Mann's methyl-blue eosin.

(1) *Schizogony*

Schizonts are distinguishable from sexual forms by being more irregular in shape and, in most instances, by lacking a clear outline (Figs. 1 and 2). Fig. 3 shows a schizont with divided nucleus, but such stages were rare in our

[1] Our thanks are due to Mr N. Mitra, Curator of the Royal Botanical Gardens, Shibpore, Bengal, who kindly supplied us with the animals.

[2] We are aware that a coccidian, *Eimeria citelli* Kartchner & Becker (1930), has been recorded from the striped ground squirrel.

preparations. Groups of merozoites were fairly common: six to eight occur in a group. We are unable to state whether there is more than one generation of merozoites. In Fig. 6 merozoites are shown escaping from a host cell into the lumen of the intestine.

Fresh material mounted in saline showed freely moving merozoites. We distinguished two types—one with hyaline cytoplasm, and the other with more opaque cytoplasm containing scattered granules. In stained smears, the hyaline merozoites measure about $6 \times 2\mu$, and they have a dark-staining granule in the neighbourhood of the nucleus: the more opaque merozoites are slightly longer, $8 \times 2\mu$, and, when stained, show numerous dark granules scattered irregularly (Figs. 7*a* and *b*). This cytoplasmic difference between the two types can be observed before the merozoites have assumed their characteristic form (Figs. 4 and 5). In the living state, the more pointed, anterior end of the merozoite moves to and fro: this region stains darkly and corresponds to the region of supposedly contractile elements described by one of us (Ray, 1935) for *Eimeria laminata*.

We believe that the micromerozoites give rise to microgametocytes, and the macromerozoites to macrogametocytes.

(2) *Sporogony*

On entering new epithelial cells, the merozoites round off and become gametocytes. It is usually possible to distinguish these sexual forms from schizonts by their definite outline; moreover, the character of their cytoplasm and its inclusions is referable to one or other of the two types of merozoite (Figs. 8 and 9). The diameter of a young gametocyte is $6–8\mu$.

Early stages in microgamete formation are rare in our preparations. Fig. 10*a* shows a portion of the periphery of a microgametocyte in which the gamete nuclei are already formed; in each protuberance of cytoplasm a basal granule lies above the nucleus. Two flagella are developed (Figs. 10*b* and *c*): in the mature microgamete one of these is directed backwards (Fig. 12). Fig. 11 shows the large number of microgametes which ultimately surround a small cytoplasmic residuum. We have observed living microgametes break from the residuum and swim actively; we have also observed them congregating at the "micropylar" end of a female gamete, in which slight agitation of the cytoplasm was then noticeable.

As the macrogametocyte increases in size, the number of granules in the cytoplasm also increases. The nucleus shifts towards one pole: its karyosome enlarges and then disintegrates.

The spherical oocyst measures $14–18{\cdot}5\mu$ in diameter. It is discharged in an unsegmented condition. Kept in 1 per cent chromic acid, it first shows sign of development after 48 hours. Fig. 16 shows four lobes projecting from the centre of the zygote; and at the tip of each of these appear four homogeneous prominences, which we believe to be the sporozoites precociously

forming. The sporoblasts do not separate from one another, however, until the fourth day: on the fifth day they develop sporocysts. There is no oocystic residue. The completed sporocyst measures $10 \times 8 \mu$, is ovoid in form, and has the characteristic lens-shaped knob at one pole described by Hoare for *Wenyonella africana*. The four sporozoites in each spore are not completely formed until the seventh day, although their presence is so early indicated. They usually lie in pairs around a sporocystic residue, and with a regular "head to tail" arrangement (Fig. 19).

(3) *Systematic position*

Our observation of the numerous, actively motile microgametes makes it clear that Hoare judged rightly in assuming that the genus *Wenyonella* should be placed in the family Eimeriidae. The species we have described differs from the type species in the following particulars: oocysts spherical, and smaller than in *W. africana*; sporozoites regularly arranged; habitat: epithelial cells of the small intestine of an Indian (Bengal) mammal, *Sciurus* sp., whereas the type species occurs in subepithelial tissues of the small intestine of an African snake.

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EXPLANATION OF PLATE VII

All figures are camera lucida drawings either of living parasites or from the smears or sections of material fixed in Bouin-Duboscq-Brasil's fluid (unless otherwise stated) and stained with Heidenhain's iron-haematoxylin. $\times 3500$, unless otherwise stated.

Wenyonella hoarei n.sp.

Fig. 1. Young schizont in an epithelial cell of the small intestine. (From a smear.) $\times 1666$.

Fig. 2. Full-grown schizont. (Section.) $\times 1666$.

Fig. 3. Schizont showing two nuclei. (Section.) $\times 1666$.

Fig. 4. Group of six female merozoites, four of which show nuclei. (Section.) $\times 1666$.

Fig. 5. Transverse section through a group of male merozoites.

Fig. 6. Group of male merozoites escaping from the epithelium. (Section.)

Fig. 7. *a*, male merozoite; *b*, female merozoite.

Fig. 8. Male gametocyte, with the characteristic dot near the nucleus. (Section.)

Fig. 9. Female gametocyte, with the characteristic granular cytoplasm. (Section.)

Fig. 10. *a*, Microgamete formation, showing pointed cytoplasmic elevations above the nuclei; *b*, a later stage, showing basal granule and flagella; *c*, more advanced microgametes. (Sections.)

Fig. 11. Group of mature microgametes around the central cytoplasmic residue. (Section.) × 1666.

Fig. 12. A mature microgamete. (Smear. Schaudinn's fluid.)

Fig. 13. Macrogamete with three male gametes adhering and one near the "micropyle", *m*. (Smear. Schaudinn's fluid.)

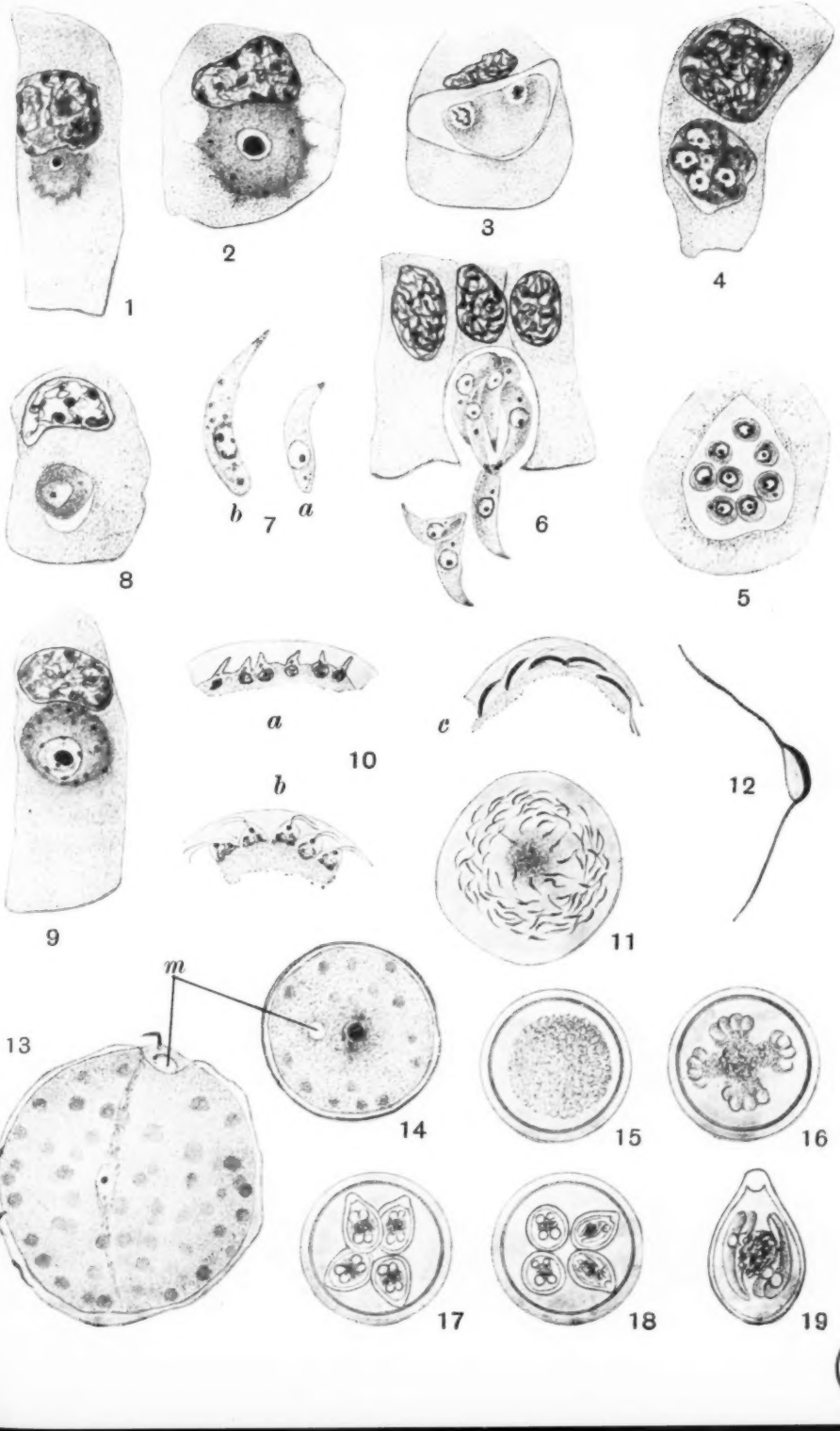
Fig. 14. Macrogametocyte, showing "micropyle". (Smear.) × 1666.

Fig. 15. Oocyst. (Living specimen.) × 1666.

Figs. 16–18. Various stages in the development of the oocyst when kept in 1 per cent. chromic acid. (Living specimens.) × 1666.

Fig. 19. Sporocyst, showing the regular arrangement of the sporozoites and the characteristic lensiform knob at one pole.

(*MS. received for publication* 15. v. 1936.—Ed.)



UNIV. OF MICH.

ON THE ASCARIDS PARASITIC IN SEALS, WITH SPECIAL REFERENCE TO THE GENUS *CONTRACAECUM*

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(With 5 Figures in the Text)

Difficulties encountered in the determination of species of *Contracaecum* from seals led the writer to undertake a comparative morphological study of a considerable amount of material. This included the Ascarids from seals collected by the Scottish National Antarctic Expedition (*Scotia*) in 1902–4, and deposited in the Royal Scottish Museum, Edinburgh, which were obtained on loan through the courtesy of Dr A. C. Stephen. In the present paper some results of this investigation are presented, and short notes are added on other species of Ascaridae recorded from Pinnipedia.

An attempt has been made to correct the names of the hosts as far as possible, and for valuable assistance in this direction the writer is indebted to Mr F. C. Fraser.

1. **Contracaecum osculatum** (Rud., 1802)

This species has been recorded not only from almost every species of seal occurring in the northern hemisphere, but also on several occasions from seals in the southern hemisphere. From the latter, however, at least two other species of the genus have been described. v. Linstow (1907) described as new species *Ascaris rectangula* and *A. radiata*, while Railliet & Henry (1907) described, also as new, *A. stenocephala* and *A. falcigera*. Leiper & Atkinson (1915) regard *A. stenocephala* as a synonym of *A. rectangula*, and *A. falcigera* as a synonym of *A. radiata*, referring both species to their genus *Kathleena*, which is now regarded as a synonym of *Contracaecum* Railliet & Henry, 1912. v. Linstow's descriptions were published in January 1907 (not in 1906, as quoted by Leiper & Atkinson), and his names have therefore six months' priority over those of Railliet & Henry.

All these authors (v. Linstow, Railliet & Henry, and Leiper & Atkinson) have recorded the species *osculatum* as occurring side by side, sometimes in the same hosts, with the species *rectangulum* (= *stenocephalum*), but it is a remarkable fact that none of them has indicated what characters enabled them to separate the two species. v. Linstow dismisses *osculatum* in a very few words, mentioning only one or two characters which are of merely generic importance, and referring to earlier descriptions of his own, the chief of which (v. Linstow,

1892) almost certainly refers, as Railliet & Henry have pointed out, to *Contracaecum radiatum* (=*Ascaris falcigera*). The only bottle of material in the *Scotia* collection determined by v. Linstow as "*Ascaris osculata*" contained some hundreds of specimens of *Contracaecum radiatum*, twenty-two specimens of *Porrocaecum decipiens* and only three which the writer regards as *Contracaecum osculatum*. Neither Railliet & Henry nor Leiper & Atkinson give any description whatever of their specimens of *C. osculatum*. The specimens recorded by the latter authors were immature. Railliet & Henry, as may be inferred from the introductory remarks to their paper, accept the account of this species given by Stiles & Hassall (1899), in whose monograph a summary of the earlier literature is given.

Now *C. osculatum* is one of the numerous "well-known" species of nematodes of which it is difficult or impossible to find adequate systematic descriptions. v. Linstow (1900), in the *Fauna Arctica*, says of it that "die Art ist oft und eingehend beschrieben", and therefore refrains from giving a description sufficient for its identification. But a study of the references quoted by him, and of the whole of the earlier literature, hardly bears out v. Linstow's statement. The descriptions of *Ascaris osculata* prior to that of Schneider (1866) may, for modern purposes, be ignored. Schneider was the first author to give any morphological details of specific value. Krabbe (1878) adds nothing to Schneider's description except that he has seen larger specimens (measuring up to 60 mm. in the male and 80 mm. in the female) and that he could not observe the ring of red pigment at the base of the lips, mentioned by Schneider. v. Linstow (1892), as has already been remarked, was apparently dealing with a different species. Jägerskiöld (1893, 1894) and v. Linstow (1895) are concerned with fine histological details rather than with gross morphology. Stossich's (1896) description appears to be derived mainly from Schneider, but the length of the male is given as 34–70 mm. and that of the female as 40–80 mm. Stiles & Hassall (1899) have given some original account of specimens from *Eumetopias jubata* [*E. stelleri*], from the Bering Sea, and it is on this description and that of Schneider that the identification of *Contracaecum osculatum* must rest.

If these descriptions be compared with v. Linstow's account of *Ascaris rectangula* and with Railliet & Henry's of *A. stenocephala*, some misgivings arise as to the distinctness of the species. Such doubts have not been dispelled from the writer's mind by the study of a considerable series of specimens from seals of various species from both northern and southern hemispheres. No constant morphological difference, in fact, has been detected between material from northern species of seals, presumed to be *Contracaecum osculatum*, and material from Antarctic seals, including the specimens identified by v. Linstow and by Leiper & Atkinson as *C. rectangulum*.

Some of the original specimens of *Ascaris rectangula* v. Linstow, obtained from *Leptonychotes weddelli* by the *Scotia* Expedition in 1902–4, are in the British Museum (Natural History). On examining these the writer found that the majority of the specimens belonged to a different genus (*Porrocaecum*) and

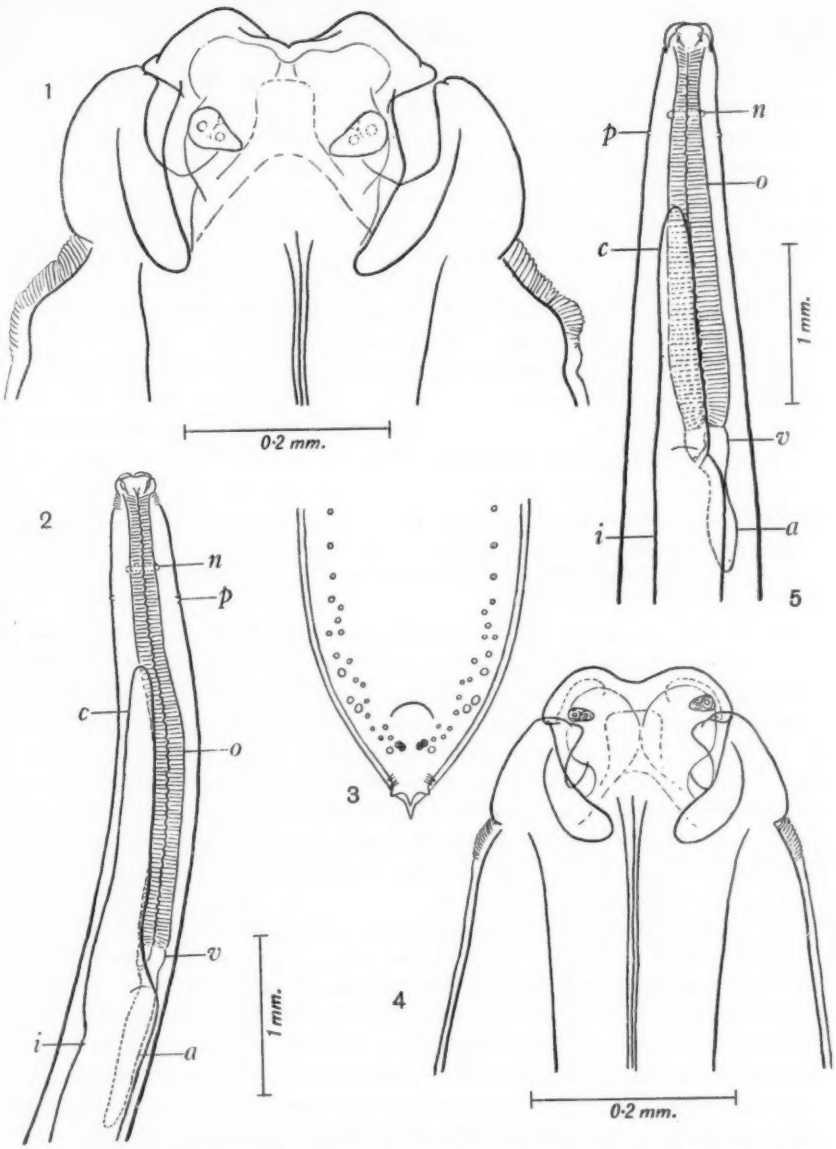

Fig. 1. *Contracaecum osculatum*. Anterior end of female; dorsal view, showing dorsal lip and two of the interlabia.

Fig. 2. *Contracaecum osculatum*. Anterior end of female; dorsal view.

Fig. 3. *Contracaecum osculatum*. Posterior end of male; ventral view, showing papillae. After Railliet & Henry ["*Ascaris stenocephala*"].

Fig. 4. *Contracaecum radiatum*. Anterior end of female; dorsal view, showing dorsal lip and two of the interlabia.

Fig. 5. *Contracaecum radiatum*. Anterior end of female; dorsal view.

a, oesophageal appendix; *c*, intestinal caecum; *i*, intestine; *n*, nerve ring; *o*, oesophagus; *p*, cervical papilla; *v*, ventriculus.

only a few were referable to *Contracaecum*. In the corresponding material in the Royal Scottish Museum the same preponderance of *Porrocaecum* was found. It is evident that the two worms were confused in v. Linstow's (1907) description of *Ascaris rectangula*, for his figure (Pl. I, fig. 1) of the dorsal lip is clearly that of a *Porrocaecum* and not of a *Contracaecum*. The study of the material supports the view of Leiper & Atkinson that the *Ascaris stenocephala* of Railliet & Henry is a synonym of *A. rectangula* v. Linstow. On the other hand, as has been stated above, no difference could be found between these specimens and *Contracaecum osculatum*, and it is therefore proposed to treat *Ascaris rectangula* and *A. stenocephala* as synonyms of that species.

In the following description an attempt is made to cover the extremes of variation met with.

Contracaecum osculatum (Rud., 1802) Baylis, 1920

(Figs. 1–3)

Synonyms. *Ascaris osculata* Rudolphi, 1802; *Fusaria osculata* Zeder, 1803; *Ascaris rectangula* v. Linstow, 1907; *Ascaris stenocephala* Railliet & Henry, 1907; *Kathleena osculata* Leiper & Atkinson, 1915; *Kathleena rectangula* Leiper & Atkinson, 1915; *Contracaecum rectangulum* Baylis, 1920.

Hosts. This species has been recorded from the following Pinnipedia in the northern hemisphere: *Cystophora cristata*, *Erignathus barbatus*, *Eumetopias jubata* [*E. stelleri*], *Halichoerus grypus*, *Monachus albiventer*, *Odobenus rosmarus*, *Phoca groenlandica*, *P. hispida* [*P. foetida*] and *P. vitulina*.[1] Its recorded hosts in the southern hemisphere (including those of its synonyms) are *Hydrurga* [*Ogmorhinus*, *Stenorhynchus*] *leptonyx*, *Leptonychotes weddelli*, *Lobodon carcinophagus*, *Mirounga* [*Macrorhinus*] *leonina* and *Otaria byronia* [*O. jubata*]. To these must be added *Arctocephalus australis* [*A. falklandicus*], specimens from this host having been sent to the writer by the late Mr A. W. N. Pillers, F.R.C.V.S., of Liverpool.

This species is very variable in size. Mature males measure about 15–70 mm. in length and 0·8–1·5 mm. in maximum thickness; mature females about 15–90 mm. and 0·8–2 mm. respectively. The head, composed of three lips and three interlabia, is narrower than the neck. The anterior cuticular border of each lip (Fig. 1) forms a pair of lobes, more or less distinctly separated by an indentation in the middle line, and produced laterally into prominent points. The free distal portion of each interlabium is more than half the total length of the interlabium. The transverse cuticular striations are at intervals of 3–8μ, being coarsest in the oesophageal region of the body. Immediately behind the bases of the lips, as in the genotype and many other species of *Contracaecum*, there is a "collar" composed of a number of deep transverse cuticular folds. There is a pair of rather large, though not prominent, cervical papillae, situated at 0·6–1·2 mm. from the anterior extremity. The oesophagus (Fig. 2, *o*)

[1] "*Phoca pantherina*" is also frequently quoted in the literature, but this name is probably a synonym of one of the species mentioned.

is of almost uniform thickness throughout, and its length, including the small posterior ventriculus, varies between 2·75 and 6 mm., according to the size of the specimen. The oesophageal appendix is usually slightly club-shaped, and measures 1–1·6 mm. in length. The intestinal caecum is 1·9–4 mm. long. The nerve ring is situated at about 0·5–1 mm. from the anterior extremity, and the excretory pore at about the same level.

The tail, in both sexes, is conical and ends in a sharp point. In the male it measures 0·15–0·3 mm. in length. The cuticle of a considerable portion of the ventral and lateral surfaces of the caudal region shows interrupted longitudinal ridges as well as transverse striations, and thus has a tessellated appearance. This may perhaps afford an explanation of Railliet & Henry's (1907) statement that the cuticle of *Ascaris stenocephala* is "striée transversalement et longitudinalement de façon à former de petits carrés de 5 à 6μ de côté". The number and arrangement of the caudal papillae have been differently interpreted by various describers. This is probably due in part to actual variation, but also in part to difficulty in seeing some of the papillae. Schneider (1866) and Stiles & Hassall (1899) observed two pairs of subventral and two pairs of sublateral papillae in the subterminal group, while Railliet & Henry found a third pair of sublateral papillae. v. Linstow's (1907) account of the papillae in *A. rectangula* is not easy to reconcile with the other descriptions, and his figure appears fantastic. One of his specimens, however, agrees perfectly with Railliet & Henry's figure (Fig. 3), and there can be no doubt that he mistook the position of the cloacal aperture. His statement that all the papillae are preanal is entirely erroneous.

The writer has seen some specimens in which the subterminal group of papillae agreed with Railliet & Henry's account, and others in which they agreed with Schneider's—i.e. sometimes three sublateral papillae could be seen on each side, and sometimes only two. It is often difficult to detect all the papillae, however, and it may be that three is the normal number. The two pairs of subventral papillae in this group appear to be constantly present, but in one specimen a third such papilla was found on the left side. As regards the more anterior papillae, all the describers are in fairly close agreement. There is constantly a pair of double subventral papillae behind the cloacal aperture, and at about the same level begins an irregular series of sublateral papillae on each side, extending forward for a considerable distance. Behind the cloacal aperture, and for some distance in front of it, these papillae are arranged in two or three irregular rows. More anteriorly they form a single row and become gradually more widely spaced apart. Usually there appear to be about fifty preanal papillae on each side. The spicules measure 5–8 mm. in length, and each is composed of a tubular shaft and a pair of alae whose edges curl inwards on the ventral side. The shaft is straight at its tip.

The tail of the female varies in length from about 0·22 to 0·55 mm., there being great variation, even in specimens of similar size, in the degree of extension or contraction of the tail. There is a pair of subterminal lateral papillae

at a distance of 0·11–0·12 mm. from the tip when the tail is well extended. The vulva is situated at about the anterior third of the body, and is usually rather prominent. The arrangement of the genital organs shows no unusual features. The eggs are subglobular and have a maximum diameter of 0·06–0·096 mm.

2. **Contracaecum radiatum** (v. Linstow, 1907) Baylis, 1920

(Figs. 4 and 5)

Synonyms. *Ascaris radiata* v. Linstow, 1907; *Ascaris falcigera* Railliet & Henry, 1907; *Kathleena radiata* Leiper & Atkinson, 1915; *Ascaris osculata* of v. Linstow, 1892.

Hosts. This species has been recorded only from seals occurring in the southern hemisphere, viz. *Leptonychotes weddelli*, *Hydrurga* [*Ogmorhinus*, *Stenorhynchus*] *leptonyx* and *Ommatophoca rossi*.

Contracaecum radiatum has been well described by Railliet & Henry (1907) under the name of *Ascaris falcigera*. The material in the Royal Scottish Museum, Edinburgh, purporting to represent v. Linstow's original specimens of *A. radiata*, proves to include no specimens answering to the description of that species, but only damaged specimens of *Contracaecum osculatum* (?) and two immature *Porrocaecum* sp. Specimens agreeing with v. Linstow's description of *Ascaris radiata* were, however, found in several other tubes of material in the *Scotia* collection.

In any case, there can be no reasonable doubt from the descriptions that Railliet & Henry's *A. falcigera* is the same species as *A. radiata*, and this is borne out by a study of specimens from the *Terra Nova* collection. v. Linstow has given a figure (1907, Pl. I, fig. 4) of the dorsal lip which, though diagrammatic, gives a fair idea of its characteristic shape.

This species does not appear to attain so large a size as *Contracaecum osculatum*. Males measure about 12–30 mm. in length and about 0·8–1·5 mm. in maximum thickness; females about 13–40 mm. and 1–2 mm. respectively.[1] The worm is distinguishable from *C. osculatum* by the following characters: The dorsal lip (Fig. 4) is relatively very wide in front and very narrow at its base, the interlabial grooves approaching much more closely to the middle line than in *C. osculatum*. The anterior cuticular lobes are less prominent, and their points are blunter and more posteriorly directed than in that species. The free distal portion of each interlabium is about half the total length of the interlabium (occasionally less than half, as indicated by Railliet & Henry, but sometimes very slightly more than half). The oesophagus (Fig. 5, *o*) is definitely club-shaped instead of being almost cylindrical. From a minimum diameter of 0·12–0·17 mm. between the base of the lips and the nerve ring, it increases fairly rapidly in diameter to a maximum of 0·28–0·45 mm. towards the posterior end. The spicules of the male measure only about 4–5 mm. in length (Leiper & Atkinson give 2·6 mm., v. Linstow 3·7 mm. (1892) or 5·13 mm. (1907)). Just before the tip of each spicule the shaft shows a characteristic bow-shaped bend (described as "en faucille" by Railliet & Henry).

[1] v. Linstow (1892) states that the male attains a length of 40 mm. and the female of 54 mm.

The caudal papillae of the male show no constant differences from those of *C. osculatum*. In one specimen no less than five sublateral papillae were seen on the left side in the subterminal group.

In addition to the two species of *Contracaecum* described above, the following other Ascarids have been recorded from Pinnipedia.

3. **Contracaecum turgidum** Chapin, 1925

Host. Monachus schauinslandii.

Locality. Hawaii (Laysan Island).

From the description of this form it appears to be closely related to *Contracaecum osculatum*, but the female attains a thickness of 2·8 mm., and the spicules of the male are very long (left spicule 11·8 mm., right 12 mm.). The form of the lips and interlabia is unfortunately not described in detail. The oesophagus appears to be of the shape of that of *C. osculatum* rather than of *C. radiatum*. The tail of the male measures up to 0·39 mm. in length, that of the female up to 0·8 mm.

4. **Phocascaris phocae** Höst, 1932

Host. Phoca groenlandica.

Locality. White Sea.

Judging from the description of this species, it represents a genus closely related to *Contracaecum*, but differing from it in having no interlabia and in the fact that the lips are provided with dentigerous ridges.

5. **Porrocaecum decipiens** (Krabbe, 1878) Baylis, 1920

Synonyms. *Ascaris decipiens* Krabbe, 1878; *Ascaris bulbosa* Cobb, 1888; *Ascaris simplex* of v. Linstow, 1888, *nec* Rudolphi, 1804, 1809.

Hosts. This species appears to be as widely distributed as *Contracaecum osculatum*. In the northern hemisphere it has been recorded from *Callorhinus alascanus* [*C. ursinus*], *Cystophora cristata*, *Erignathus barbatus*, *Eumetopias jubata* [*E. stelleri*], *Halichoerus grypus*, *Mirounga angustirostris*, *Odobenus rosmarus*, *Phoca groenlandica*, *P. hispida*[1] [*P. foetida*] and *P. vitulina*. Some specimens, apparently of this species, from *Monachus albiventer* from Senegal are in the collection of the British Museum (Natural History). The hosts of *Porrocaecum decipiens* in the southern hemisphere appear to include *Arctocephalus gazella*, *Leptonychotes weddelli* and *Ommatophoca rossi*.

v. Linstow (1888) recorded "*Ascaris simplex* Rudolphi" from "*Otaria jubata*" obtained by the *Challenger* at Kerguelen, 27 January 1874. The *Challenger* specimens were redetermined by Baylis (1916) as *Ascaris* (now *Porrocaecum*) *decipiens* Krabbe. *Eumetopias* [*Otaria*] *jubata* does not, however,

[1] *Phoca largha*, recorded as a host for this species by Stiles & Hassall (1899) is probably a synonym either of *P. hispida* or of *P. vitulina*.

occur at Kerguelen. The *Challenger Report* on seals, by Sir William Turner, refers to two carcases of young fur-seals procured at Fullers Harbour, Kerguelen, in January 1874. These are referred to *Arctocephalus gazella*, which is therefore probably the host of the specimens of *Porrocaecum decipiens*. Railliet & Henry (1907) record this species from *Leptonychotes weddelli* and *Ommatophoca rossi*. It was also the most abundant form in the material from these two hosts obtained by the *Scotia* expedition, as evidenced by the material in the Royal Scottish Museum, and it is remarkable that the species is not mentioned by v. Linstow (1907) in his report on that collection. As has been noted above, however, v. Linstow figured the dorsal lip of what was probably *Porrocaecum decipiens* as that of "*Ascaris rectangula*".

A good description of *Porrocaecum decipiens* has been given by Stiles & Hassall (1899), to which some details were added by Baylis (1916).

6. **Anisakis similis** (Baird, 1853) Baylis, 1920

Synonym. *Ascaris similis* Baird, 1853.

Hosts. This species was originally recorded from an unnamed species of seal from the Antarctic regions. It has since been recorded (Baylis, 1929) from the sea-elephant (*Mirounga leonina*) at South Georgia. There are also specimens in the British Museum (Natural History) from "*Macrorhinus ursinus*" (probably *Mirounga leonina*) from the Falklands. These appear to have been determined by v. Linstow in 1899 as "*Ascaris patagonica*" (*q.v., infra*). Further, some specimens, mainly immature, from a grey seal (*Halichoerus grypus*) taken off Cornwall in 1933, have been referred by the writer to *Anisakis similis*.

For a redescription of Baird's original specimens of *A. similis* see Baylis (1916).

7. **Anisakis rosmari** (Baylis, 1916) Baylis, 1920

Synonyms. *Ascaris bicolor* Baird, 1868, *nec* Rudolphi, 1793; *Ascaris rosmari* Baylis, 1916; *Anisakis alata* Hsü, 1933.

Host. Walrus (*Odobenus rosmarus*).

Baird's original specimens of *Ascaris bicolor* were redescribed by Baylis (1916). The fact that the writer failed to describe the second pair of postanal papillae of the male as having double nerve-endings appears to have led Hsü (1933) to suspect that he was dealing with a different species. Re-examination of the original material shows, however, that these papillae have double terminations, while there is great variation in the relative sizes and positions of the papillae forming the posterior group, to which Hsü has also drawn attention. Hsü's specimens were smaller than the types, but the writer has no hesitation in regarding *Anisakis alata* as a synonym of *A. rosmari*.

8. "**Ascaris**" **patagonica** v. Linstow, 1880

This very inadequately described form is recorded by v. Linstow (1880) from "*Otaria jubata*" (probably *O. byronia*) from Patagonia. As v. Linstow seems later to have referred specimens of *Anisakis similis* (*q.v., supra*) to this species, it seems possible that it may be a synonym of *A. similis*. His figure of the dorsal lip of *A. patagonica* is, however, difficult to reconcile with this view.

9. "**Ascaris**" **dehiscens** v. Linstow, 1905

This form, recorded from *Phoca hispida* [*P. foetida*], appears to have been immature, and is probably unrecognizable from the description. No characters are mentioned by which it can be assigned to a genus, and the figure of the head is not very intelligible.

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(*MS. received for publication* 7. v. 1936.—Ed.)

EXPERIMENTAL WORK ON REPRODUCTION IN THE TSETSE FLY, *GLOSSINA PALPALIS*

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(With 2 Figures in the Text)

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Introduction

The experiments described in this paper were carried out during a visit to Uganda in 1935. The work was done at the Human Trypanosomiasis Institute at Entebbe, and I am greatly indebted to the Director, Dr H. L. Duke, for his hospitality and encouragement.

The flies used in these experiments were obtained from wild pupae collected from the shores of Lake Victoria by native "fly boys". Within 24 hours of the flies emerging from their pupae they were separated into batches of males and females. They were placed in "Bruce"[1] boxes or celluloid cylinders with gauze ends. Almost all the flies were offered food daily by the box or cylinder being fixed down against the side of a chicken. The individual flies in the section on nutrition were fed on man.

The object of this work was to find out the effect of temperature, humidity and nutrition on the reproductive cycle of the female fly.

The section on nutrition and factors governing larviposition was written in collaboration with Dr Kenneth Mellanby.

[1] A Bruce box is made of wood—size about 15 × 10 × 7·5 cm. with gauze across the two largest sides.

THE NORMAL REPRODUCTIVE CYCLE AT AN AVERAGE TEMPERATURE OF 24° C.

The main features of the reproductive cycle of the genus *Glossina* are well known. All the species are viviparous and produce fully grown larvae, which pupate within a few hours of birth. Descriptions and diagrams of the female reproductive system are given by Minchin (1905), Roubaud (1909), and Newstead (1924), and a comparison of viviparity in *Glossina* with that of other

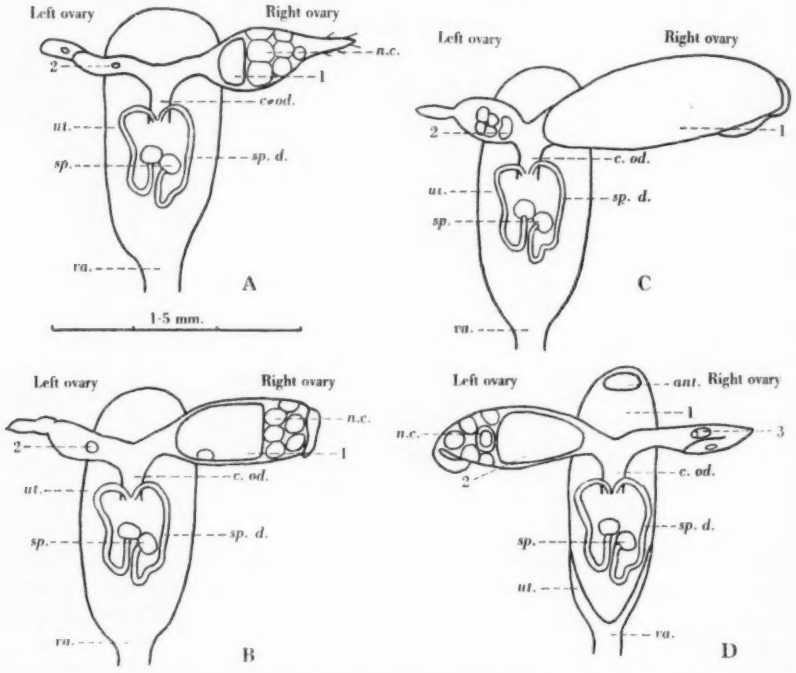

Fig. 1. Four stages in the development and ovulation of the first egg in *Glossina palpalis*. A, ♀ under 24 hours old, rt. egg beginning to develop. B, ♀ 4 days old, rt. egg about one-third developed. C, ♀ 7 days old, rt. egg fully developed. D, ♀ 16 days old, rt. egg ovulated for less than 24 hours. 1 = 1st egg to develop fully; 2 and 3 = second and third eggs. *ant.* = anterior end of ovulated egg. *c.od.* = common oviduct; *n.c.* = nurse cells; *sp.* = spermatheca; *sp.d.* = spermathecal duct; *ut.* = uterus; *va.* = vagina.

Diptera is given by Keilin (1916). The chief features of the reproductive system are, first, the ovaries, which each consist of one polytropic ovariole. Only one egg ripens at a time from alternate ovaries. Each ovary connects by means of an oviduct with a short common oviduct, which leads into a uterus (Fig. 1). The accessory glands are modified to produce a secretion on which the developing larva lives. On the dorsal wall of the common oviduct open the spermathecal ducts leading from the spermathecae. The eggs are fertilized in the uterus,

and according to Roubaud (1909) the embryonic period lasts 3 days, and the larval period 4 or 5 days. The larva undergoes three moults. The first takes place soon after hatching, the second shortly before the larva is born, and the third as the larva pupates. An individual fly can produce up to eight larvae at about 10-day intervals in the laboratory (Roubaud, 1909).

The development of the ovaries and ovulation at an average temperature of 24° C. *and about* 70 *per cent* R.H.

Flies were dissected by cutting a thin slice off each side of the abdomen, from the second segment to the level of the sixth segment. Then a cut was made along the anterior margin of the second tergum, care being taken to avoid injuring the abdominal contents. The dorsal wall of the abdomen could then be reflexed, leaving the contents undamaged and undisturbed.

In newly emerged female flies the ovaries are immature (Fig. 1 A), but the lowest ovum of the right ovary is invariably more advanced than that of the left. (Many hundreds of dissections did not reveal a single example of the left ovary developing first.) Females were kept at a mean temperature of 24° C. (varing between 21 and 26° C.), and batches were dissected daily. The first egg in the right ovary is not fully developed until the 7th day (Fig. 1 B, C), and fertilization of the female did not affect this rate of development. Ovulation was never observed earlier than the 8th day after emergence in many hundreds of flies, and it frequently occurred later. After the first egg is ovulated, the second develops (Fig. 1 D), becoming full-sized in about 8 days, by which time the uterus contains an advanced larva.

Virgin females were dissected at daily intervals from the 8th to the 21st day after emergence, and in practically every case the first egg was not ovulated, but remained in the right ovary fully developed. The second egg (the lowest egg in the left ovary) seemed to develop more slowly than in the case of the fertilized fly where the first egg had been ovulated; it was fully developed by the 21st day. Virgin females 4 or 5 weeks old were found to contain three or four fully developed eggs, but in about a third of the flies examined one or more eggs had been ovulated, and were found in the uterus. This ovulation is most probably mechanical, due to the development of the succeeding ovum in the same ovariole. None of these ovulated eggs was found "extruded", though probably that was what would have happened to them.

In order to be sure of having fertilized females for these experiments, it was necessary to put numbers of females of known age into a cage with potent males, and remove pairs in copula to separate tubes. If they did not separate for at least half an hour, then the spermathecae of the female was certain to contain sperms (K. Mellanby, 1936). If males and females are merely kept in the same cages, some females will remain virgin for weeks and complicate the experiments.

Ovulation of the first egg was found to occur between the 1st and 11th day after fertilization of the female; it usually took place during the first 3 days,

and it was only once observed as late as the 11th day. Ovulation is, therefore, definitely connected with fertilization, though there was individual variation in the time taken to ovulate the first egg. Mackerras (1933), working with blow-flies, found that an adequate amount of protein was required in the diet of the female before the eggs would develop. He also states that "copulation appears to provide some essential stimulus for oviposition". Roubaud (1909) insists that ovulation in *Glossina* is not mechanical, but that it is controlled by the nervous system.

The second and succeeding eggs in fertilized females were found by dissection to be ovulated within 24 hours of the production of the previous larva. The egg began to descend the oviduct within 15–18 hours. Irregularities in the time of ovulation such as occur with the first egg were never found with the succeeding ones.

The length of embryonic and larval life

When it was realized that ovulation of eggs after the first happens so regularly, it became possible to find the length of embryonic and larval life by dissecting female flies at definite times after producing a larva. The embryonic period lasts for $3\frac{1}{2}$ days, and the larval period for 5 days. The average period between the births of two successive larvae at this temperature was 9·9 days, the shortest being 9 days and the longest 11 days out of a total of 27. The average weight of the larvae produced in the laboratory and which were able to pupate was 26·9 mg.

The delay in ovulation of the first egg, and the fact that the first egg is never ripe before the 8th day, explains why the period taken to produce the first larva is long and irregular compared with later larvae. The delay is not due to the accessory glands being undeveloped as suggested by Roubaud (1909). In cultures where males and females are merely kept together, delay in fertilization of some females may make the period more variable still. Once the first larva has been produced the process continues with surprising regularity.

Nutrition and reproduction[1]

It has often been observed by various workers that the pupae of tsetse flies, which are produced in the laboratory, are on the average smaller than those found in the field. This is the case for *G. morsitans* and *G. swynnertoni* (Potts, 1936). Roubaud, working with *palpalis*, must have usually had very small pupae produced in captivity, for he comments on "une larve très volumineuse" of 25 mg., whereas wild pupae of this species weigh nearly 35 mg. when produced. Our average weight for larvae produced in the laboratory was 26·9 mg. Buxton & Lewis (1934) found that many laboratory pupae of *G. tachinoides* and *G. submorsitans* were undersized.

We thought of a number of possible explanations which would account for these small larvae and pupae. At first we supposed that the cramped conditions

[1] This section was written in collaboration with Dr Kenneth Mellanby.

or the particular light intensity of captivity made the female larviposit earlier than it would in the field, and so produce a small pupa. This seemed reasonable, because so many muscid larvae are able to pupate when they are not fully grown if the conditions of feeding are unfavourable. But experiments using larger cages and many different light intensities gave results indicating that these factors had little effect. The mean weights of the pupae produced by isolated females in large cages were the same as that of others which were kept several together in a small container. The presence or absence of males did not affect the pupal size. (It has been suggested that the presence of males might make the females larviposit early.) Flies which were kept in the light during the day produced pupae of the same size as did those kept in the dark all the time, except when being fed.

When we observed that the period of time taken to produce successive larvae after the first was so constant the problem became easier. Larvae were produced after a definite time at any one temperature, and such larvae varied greatly in size. Clearly then the smaller larvae (down to 20 mg. in weight) were not necessarily small because they had been deposited early. A larva weighing 25 mg. took exactly as long to produce as did one of 35 mg.

It seemed probable that the weight of the larva might depend on the amount of blood taken during pregnancy. To test this idea, a number of female flies which had just produced their first pupae, were isolated in individual tubes, and a record made of the weights of blood which they took during the next pregnancy. They were kept at 24° C. Each day they were weighed, offered a meal, and if they fed they were weighed again. It was found that on the whole the more blood a fly took during the period of gestation, the larger the final

Table I. *The relation between the weight of blood taken during pregnancy and the weight of the larva produced.* 24° C.

Range of larval weights in each batch mg.	Average weight of larvae in each batch mg.	Total blood taken (averages)		Numbers of larvae in each batch
		Mean wt. mg.	S.E.	
Below 25	23·3	159·7	4·62	10
25–30	27·4	178·3	7·12	12
Above 30	33·6	218·4	8·63	7

larva and therefore the heavier the pupa. The results of this experiment are summarized in Table I.[1] The total weight of blood taken appeared to be slightly less important than the time at which the meals were taken. Three really large meals were sufficient to give a pupa weighing as much as a wild one. The females producing the largest pupae all took a really good meal 3 days or so before producing their larva. At that time they were able to accommodate a large amount of blood in their crops without interference from the developing larva, since the larva was then very small. In the laboratory it is not easy to make the flies feed at the right times, so that it is difficult to get many large

[1] These figures give a correlation coefficient of +0·802, which shows that there is a high degree of relationship between the amount of blood taken and the size of the larva produced.

pupae. If a fly is fed too often, it cannot take a large meal at the correct time. On the other hand, a fly may refuse to feed at all, although it must be hungry.

Now it seems that if nutrition determines the size of larvae, then the frequent "abortions" of partly grown larvae observed in laboratory cultures of the fly may be due to the failure of the flies to obtain sufficient nourishment. It was observed that females which died from starvation seldom contained a larva, and if a culture was allowed to die out, then there were numerous "abortions" before the flies died. A number of experiments were carried out to investigate this point.

In the first experiment fourteen newly fertilized females 7–8 days old were isolated in separate tubes, and fed on alternate days. At each meal the flies were only allowed to take 10–15 mg. of blood instead of a full meal of between 50 and 60 mg. The flies survived well and there were no deaths in 21 days, but there were no fully developed larvae produced, only a few "aborted" larvae weighing 2 or 3 mg. Seven of these flies were dissected at the end of 21 days, and it was possible to tell from the state of their ovaries that one or two eggs had been ovulated in every case (see p. 133 above). These eggs were either extruded before the larva hatched or shortly after. Some flies had newly hatched larvae in their uteri at the time of dissection. Not all the extruded eggs were observed in the tubes as they are very small (about 1·4 mm. long) and easily covered up by excrement. The remaining seven flies were allowed to have large meals after the 21st day, and when they were dissected on the 28th day they were found to contain large larvae. This experiment was repeated in London, when seven females were given partial feeds for 24 days. During that time five extruded eggs were observed and one very small larva. Sections of the eggs showed that the embryo was not in the same stage of development in each case. Eight other females, which were kept as controls and allowed to take as much blood as they liked, produced during the same time ten larvae (all of which pupated) and one small aborted larva. When the partially fed flies were allowed to gorge they produced large larvae. Fig. 2 shows the change in weight of two flies, one of which fed well and produced a large larva, while the other had its meals interrupted and aborted a tiny larva.

In the next type of experiment six older females, which had previously produced at least one larva, were used. They were given small meals for 14 days. During that time no full-grown larvae were produced, but three eggs and three very small larvae were extruded. This showed that the normal reproductive cycle (see p. 134) was completely interrupted if the flies were insufficiently nourished. The ovaries of some partially fed females were sectioned, and the oocytes were found to be developing normally.

These experiments show that the female of *Glossina palpalis* is unable to produce sufficient nourishment for the development of her larva unless she is herself adequately fed. Severe shortage of food results in the early extrusion of eggs or larvae. Less severe starvation allows the larva to remain in the uterus for the normal time, but it does not allow full development. Buxton & Lewis

(1934) found that they obtained larger larvae at 23° C. than at 30° C., though the weights were very variable. They also observed that the flies which emerged from wild pupae in the cool season were heavier than those which emerged in the hot. We state above that in captivity really large larvae are

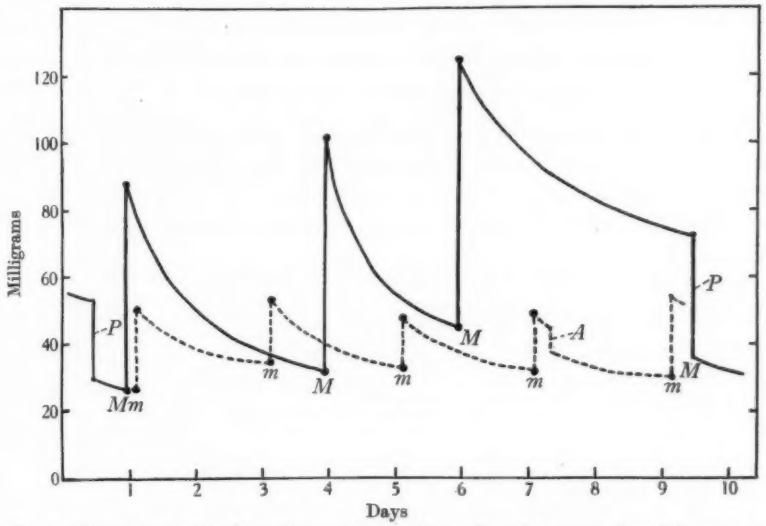

Fig. 2. Change in weight of two flies, continuous line = allowed to gorge, dotted line = meals interrupted. *M.* = a full meal, *m.* = an interrupted meal, *P.* = pupa, *A.* = abortion.

produced when the female takes a large meal at just the right stage in gestation. Now the lower the temperature the longer is the period of gestation, and the greater the chance that the fly will take a large meal at the correct time. We found that flies kept at a low temperature (below 22° C.) produced pupae slightly above average weight for captivity. The great variation in size found under all laboratory conditions supports the view suggested above. Wild flies produce pupae of a more uniform weight because they are able to feed when they become hungry and are not stimulated by food too often. When food is scarce in the field, wild flies may well abort their larvae as do captive ones. Johnson & Lloyd (1923) found a close relation between the amount of food in the gut of wild female *G. tachinoides* and the number of flies which were pregnant. Many more flies had recently fed, and were pregnant during the hot dry season than during the wet season.

FACTORS GOVERNING LARVIPOSITION

Under controlled conditions the embryonic and larval periods have been shown to be very uniform. It was observed in the laboratory that the exact time of larviposition was affected by changes in temperature, though it was quite unaffected by the presence or absence of light. Larvae were always

produced in the hotter hours of the day and never at night. On the other hand, a reduction of the temperature to 20° C. could only delay larviposition in a female which was ready to produce a larva for about 12 hours. This agrees with the observation of Carpenter (1911), that in the field females only larviposited during the warmer hours of the day.

Effects of different temperatures on the cycle

Higher temperature than normal (30° C.)

Boxes of flies containing males and females were placed in an incubator run at 29–30° C., 1, 6, 7 and 10 days after the flies had emerged from their pupae. They had previously been kept under laboratory conditions of temperature. These flies were offered food every day and every effort was made to see

Table II. *Effect of* 29–30° C. *on mixed cultures of tsetse flies. Experiment lasting* 28 *days*

No. of box	Age of flies when placed at 29–30° C. days	No. of flies ♀	No. of flies ♂	Fertilized ♀ which died showing abnormal ovaries on dissection*	♀ surviving 28 days	♀ surviving which were fertilized	Ovaries abnormal (in surviving females)
1	1	29	34	4/4	2	—	—
2	10	18	26	4/4	6	6	6
3	7	33	51	1/1	11	10	9
4	6	25	30	4/6	12†	(11)	(12)

* Many decayed, could not be dissected.

† Transferred to laboratory conditions on 28th day, and kept there for 21 days. No larvae produced, and all were abnormal when dissected. Ovaries had been permanently upset.

that they fed. During the first few days there were a good many deaths, then the flies appeared to settle down to the new conditions and they lived fairly satisfactorily. When females were found dead they were dissected if they had not decayed too much. After 28 days not a single fully or partially developed larva had been produced in any of the boxes. The remaining females in three of the boxes were then dissected. The flies in the fourth box were kept for a further 21 days at laboratory temperature to see if they would then produce any larvae. No larvae were produced from flies in the fourth box during that time. The results of the dissections are given in Table II. It will be seen that a very high proportion of the females were fertilized (i.e. they had sperm in their spermathecae), but nearly all of them showed abnormalities in their ovaries. Either the ovaries contained several ova fully developed in one ovary (cf. old virgin flies) or there were no visible ova. A few days' exposure to a constant high temperature would therefore appear to induce permanent sterility by upsetting ovulation and the development of the ova.

The next experiments were carried out on females which were known to have produced at least one larva. Twelve such females were taken immediately after the production of a larva and kept under laboratory conditions of

temperature. They were then transferred to the 30° C. incubator after they had been 1, 2, 3, 4 and 5 days at laboratory temperature. Of these flies five only produced larvae (four of which pupated). Three of the flies which produced larvae were placed at 30° C. on the 4th day and the other two on the 5th day (by which time the egg would have hatched). All the flies placed at 30° C. earlier than the 4th day failed to produce larvae by the 10th day and were then dissected. It was found that in these seven flies the egg remained unhatched in the uterus, so 30° C. must be unfavourable for embryonic, though not for larval, development. Most insect eggs develop normally at 30° C., or even a few degrees above, including those of other tsetse flies (Buxton & Lewis, 1934, working with *G. tachinoides* and *submorsitans*).

Lower temperature than normal (below 22° C.)

A number of females were kept, together with males, in an incubator in saturated air at a temperature which did not exceed 22° C. Ten were dissected on the 10th day, and the ovary was in the stage of development shown in Fig. 1 B. A further six females were dissected on the 12th day, when the first egg was found to be fully developed (Fig. 1 C). This low temperature causes the egg to take about 11 days to become fully developed, while the egg develops in 7 days at 24° C.

When boxes containing both males and females were placed in the cold incubator, a long period elapsed before the first larva was produced. In one box containing twenty-eight females and twenty-nine males the first larva was produced in 37 days, and only nineteen were produced in 75 days. Thirteen females survived, and on dissection twelve were normal, seven having larvae in their uteri. The thirteenth had not been fertilized. In other boxes a lower percentage of females were fertilized. This was because at 22° C. *Glossina palpalis* is very inactive, so the small number of females fertilized and the low temperature both helped to reduce the productivity of the cultures.

Twenty-five females were kept under room conditions until fertilization was observed, and they were then placed in the cold incubator. Twenty of these which were isolated in $2 \times 1\frac{1}{2}$ in. tubes produced their first pupae between 13 and 26 days after fertilization (average $16\frac{1}{2}$ days). Eight second pupae were produced in an average time of $17\frac{1}{2}$ days (one in 16, six in 17, one in 22 days[1]). Dissections of the twenty-one surviving females after the experiment had lasted for 42 days showed that eighteen were normal, and three had developed abnormally after producing one pupa.

Field conditions

Five females which had produced one or more larvae previously, were placed in a net cage in the grounds of the laboratory immediately after they had deposited their previous larva. They produced their next larva in an average

[1] During the period when the first larvae were produced, the ice supply was erratic, and the temperature fluctuated. Probably the average period for the first larvae should be rather longer.

time of 13½ days (shortest 12½ days, longest 14 days). This experiment was done during December and the flies were subjected to conditions closely approximating those found in nature.

The effect of different humidities on the cycle

Batches of ten males and ten females were put into celluloid cylinders on the day of their emergence from pupae. Three such cylinders (each containing twenty flies) were placed in desiccators at 88 per cent R.H. and 47 per cent R.H. Three more were kept under laboratory conditions of humidity (about 70 per cent R.H.) as controls. Flies will live well at 47 per cent R.H. if they are very carefully fed.

After 34 days the experiment was stopped, and the remaining eleven females at 47 per cent and twelve females at 88 per cent were dissected. All the females from the 47 per cent were normal and fertilized. Three of those at 88 per cent were abnormal and two of these were unfertilized. At 47 per cent R.H. thirty-nine larvae were produced of average weight 27 mg. At 88 per cent R.H. nineteen larvae were produced of average weight 26·3 mg., and there were four observed abortions. The controls produced twenty-one larvae. It appears from this that a low humidity is not unfavourable, but it must not be assumed that the figures for the number of larvae show that a low humidity is particularly favourable. Very great care was taken with the flies kept at 47 per cent R.H. to ensure that they all fed (since they died very readily at this humidity from desiccation (K. Mellanby, 1936)). This resulted in their being better fed than the others, and so producing more larvae and fewer abortions. The low-temperature experiments showed that even saturated air does not have any harmful effect on reproduction (p. 139 above).

Summary

1. The reproductive cycle of the female fly was worked out at an average temperature of 24° C. The lowest egg of the right ovary develops first, and is ripe in 7 days. Ovulation never occurs earlier than the 8th day, and frequently later; it bears some relation to fertilization, for eggs in virgin females failed to ovulate for weeks. The first larva was produced in a variable time after fertilization (because of delayed ovulation), but the succeeding larvae were produced very regularly, with an average period of 9·9 days between them.

2. Pupae produced in the laboratory usually weigh less than those collected in the field. The reason for this is nutritional, and flies must take large meals at the right stage in gestation to produce large pupae. It is impossible to ensure that this happens with all flies in the laboratory.

3. Abortions are caused in captivity by flies obtaining too little blood. They may be induced in pregnant females by allowing them to take only small meals, when either an egg or a small larva is extruded. When these flies were properly fed later, they produced normal larvae.

4. A constant temperature of 30° C. causes sterility in females. Ovaries develop abnormally, embryos fail to hatch from eggs, but young larvae (in the uteri) are not adversely affected.

5. A constant temperature below 22° C. slows down the rate of development of the eggs in the ovaries and lengthens the larval period. Experiments at this temperature are complicated by inactivity of males, which leave many females unfertilized. The length of the combined embryonic and larval periods is 17½ days.

6. Experiments performed outside in the laboratory compound suggest that the period between each larva in the field (during December in Uganda) will be about 13½ days (min. temp. 16° C.; max. temp. 27° C.).

7. Flies appear to be able to breed equally well with relative humidities of 47, 88 and 100 per cent. In the drier air care must be taken with feeding, or the flies die from desiccation.

8. A temperature of 20° C. can cause pregnant females to retain their larvae for an extra 12 hours, but not longer.

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(*MS. received for publication* 21. v. 1936.—Ed.)

THE REPRODUCTIVE CYCLE IN *GLOSSINA MORSITANS* AND *GLOSSINA SWYNNERTONI*

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During a visit to Tanganyika, thanks to the kindness and help of the Department of Tsetse Research, I was able to investigate the reproductive cycles in *Glossina morsitans* and *G. swynnertoni*, and compare them with the cycle in *G. palpalis*. In the previous paper in this *Journal*, Mrs H. N. Mellanby gives a detailed description of her work with *G. palpalis*, and the following comments on the similarity of the other species is all that is required.

(1) In both *G. morsitans* and *G. swynnertoni*, when the female emerges from the pupa the ovary is practically identical with Fig. 1 A in the foregoing paper.

(2) Examinations of several hundred dissections showed that the right ovary always develops first, and that the rate of development at a mean temperature of 24° C. is practically the same for all three species.

(3) In all species, if the female has been fertilized it is very easy to see the sperm inside the spermatheca by dissecting it out and examining it in normal saline. No sectioning is needed (see Mellanby, K., 1936).

The main significance of these results is that these three different species should behave almost identically; it is therefore probable that all species of tsetse have a similar reproductive cycle. This information may be of some importance in assisting field investigations of the reproduction of the various species.

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(*MS. received for publication* 3. vii. 1936.—Ed.)